HISTAMINE RECEPTOR 4 (H_4R) IN PATHOGENESIS OF SJÖGREN'S SYNDROME

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«A delusion is something that people believe
in despite a total lack of evidence».

- Richard Dawkins, evolutionary biologist

«Knowing the answer means nothing;
Testing your knowledge means everything».

- Lawrence Krauss, theoretical physicist and cosmologist

Dedicated to humanity

In memoriam

Professor Yrjö T. Konttinen

Photo by Erkki Hänninen

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which in the text are referred to by Roman numerals (I – III)

I **Stegaev V**, Sillat T, Porola P, Hänninen A, Falus A, Mieliauskaite D, Buzás E, Rotar Z, Mackiewicz Z, Stark H, Chazot PL, Konttinen YT. Brief report: first identification of H_4 histamine receptor in healthy salivary glands and in focal sialadenitis in Sjögren's syndrome. Arthritis Rheum. 2012 Aug;64(8):2663-8. doi: 10.1002/art.34484.

II **Stegaev V**, Nies AT, Porola P, Mieliauskaite D, Sánchez-Jiménez F, Urdiales JL, Sillat T, Schwelberger HG, Chazot PL, Katebe M, Mackiewicz Z, Konttinen YT, Nordström DCE. Histamine transport and metabolism are deranged in salivary glands in Sjögren's syndrome. Rheumatology (Oxford). 2013 Sep;52(9):1599-608. doi: 10.1093/rheumatology/ket188.

III **Stegajev V**, Kouri VP, Salem A, Rozov S, Stark H, Nordström DC, Konttinen YT. Activation of histamine H4 receptor inhibits TNFα/IMD0354-induced apoptosis in human salivary NS-SV-AC cells. Apoptosis. 2014 Dec;19(12):1702-11. doi: 10.1007/s10495-014-1036-6.

In addition, some unpublished results are presented and designed as **IV**.

2. LIST OF ABBREVIATIONS

AMP	antimicrobal peptides
ANOVA	analysis of variants
APC	antigen-presenting cell
AQP	aquaporin
ATP	adenosine triphosphate
BAFF	B-cell activating factor
BAX	Bcl-2-associated X protein
Bcl-XL	B-cell lymphoma-extra large protein
Bcl	B-cell lymphoma
Bid	BH3 interacting domain death agonist
BPE	bovine pituitary extract
BSA	bovine serum albumin
CD	cluster of differentiation
CNS	central nervous system
cPARP	cleaved poly (ADP-ribose) polymerase
CXCL	chemokine (C-X-C motif) ligand
DAB	3,3'-diaminobenzidine
DAO	diamino oxidase
DAPI	4',6-diamidino-2-phenylindole
cDNA	complementary deoxyribonucleic acid
Do, Go, Rb	donkey, goat, rabbit
ECL-cell	Enterochromaffin-like cell
ELISA	enzyme-linked immunosorbent *assay*
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FADD	Fas-assosiated death domain
FAM	fluorescein amidite
FasL	Fas-ligand
FasR	Fas-receptor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GM-CSF	granulocyte macrophage colony-stimulating factor
GPCR	G-protein-coupled receptor
GTP	guanosine triphosphate
H_1R	H1 histamine receptor
H_2R	H2 histamine receptor
H_3R	H3 histamine receptor
H_4R	H4 histamine receptor
HDC	histidine decarboxylase
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HNMT	histamine-N-methyl transferase
HRP	horseradish peroxidase

hrTNFα	human recombinant tumor necrosis factor alpha
HSG cell	derivative from human HeLa cells
hY RNA	human non-coding small ribonucleic acid
IF	immunofluorescence
Ig	immunoglobuline
IHC	immunohistochemistry
IL-	interleukin
IFN	interferon
JNK	c-Jun signalling kinase
K-SFM	keratinocyte serum-free medium
Kd	dissociation constant
LSG	labial salivary gland
M3 receptor	muscarinic receptor 3
MAC	apoptosis-induced channel
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
moH4R	murine/mouse histamine receptor 4
MPP	1-methyl-4-phenylpyridinium
mRNA	messenger ribonucleic acid
MβCD	methyl-β-cyclodextrin
NF-κB	nuclear factor κB
NK-cell	natural killer cell
NO	nitric oxide
NOD mice	non-obese diabetic mice
Noxa	phorbol-12-myristate-13-acetate-induced protein 1
NSAID	nonsteroidal anti-inflammatory drugs
NS-SV-AC	normal salivary simian virus-40 immortalizes acinar cells
OCT	organic cation transporter
PBS	phosphate-buffered saline
PI	propidium iodide
PMAT	plasma membrane monoamine transporter
PPI	proton pump inhibitors
pSS	primary Sjögren's syndrome
Puma	p53 upregulated modulator of apoptosis
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative reversed transcriptase polymerase chain reaction
R-MeHA	R-methylhistamine
SCC	squaomous cell carcinoma
SEM	standard error of mean
SMG	submandibular gland
SS	Sjögren's syndrome
SSA/Ro	anti-Sjögren's-syndrome-related antigen A
SSB/La	anti-Sjögren's-syndrome-related antigen B
sSS	secondary Sjögren's syndrome
TBS	tris-buffered saline
TGX	tris-glycine eXtended

T_h cell	T-helper cell
THIO	thiopyrimidine
TLR	toll-like receptor
TNFR1	TNF receptor 1
TRADD	TNF receptor-associated death domain
TTBS	Tween-20 Tris-bufferes saline
VEGF	vascular endothelial growth factor
VMAT	vesicular monoaminotransporter

3. ABSTRACT

Introduction. Sjögren's syndrome (SS) is a chronic autoimmune disease with yet unknown etiology and partly unclear pathogenesis that affects exocrine glands. Cardinal symptoms of the disease are dry mouth and eyes. Fas-apoptosis is considered as a key event in breach of autoimmune tolerance in SS. Increased levels of histamine in serum and tissue fluids characterize many autoimmune diseases, including SS. However, trials have showed ineffectiveness of H_1 and H_2 histamine receptor antagonists in the treatment of autoimmune diseases. More recently, it has been reported that many cells throughout the human body synthesize histamine in small nanomolar quantities in a non-professional manner. Unlike professional histamine-synthesizing, -storing and stimulus-mediated burst-like -releasing cells, such as mast cells, basophils and ECL-cells, many other cells, including dendritic- and T cells can synthesize histamine at 100-1000-fold lower rate and release it continuously into the extracellular milieu. In the beginning this discovery drew little attention until a new H_4 receptor (H_4R) was discovered in immune cells in 1994. H_4R has about 10000-fold higher affinity to histamine than conventional H_1R and H_2R. This discovery shortly became a hot topic and started a new era for studies of immunomodulatory histamine/H_4R-mediated effects. Today clinical trials of H_4R antagonist as treatment for inflammatory diseases are ongoing.

Aims of the thesis project. It was hypothesized that H_4R is involved in SS pathogenesis. To test this hypothesis the following objectives were studied: (1) expression of H_4R in salivary glands of healthy and SS individuals; (2) H_4R internalization and functionality upon specific agonist stimulation; (3) transport of histamine in HSG cells and expression of histamine metabolizing enzymes in salivary glands of healthy and SS individuals; (4) mechanisms of anti-apoptotic activity of H_4R; (5) mRNA levels of moH_4R in orchidectomized and intact male mice.

Materials and methods. Briefly, this study was performed on snap-frozen and/or paraffin-embedded biopsies of minor salivary glands from SS patients, which were routinely taken as a part of the diagnostic procedure. Samples were considered as "healthy" if diagnostic criteria were not fulfilled. *In vitro* experiments were performed on two cell lines: human salivary gland (HSG) line, which is phenotypically a ductal

epithelial line, and normal salivary Simian virus 40-immortalized acinar cell (NS-SV-AC) line. Additionally, we were gifted by snap-frozen murine salivary glands from BALB/c, NOD, orhydectomized and intact HDC^{+}/HDC^{+} lines. Protein expression levels of histamine receptors, transporters and metabolizing enzymes in tissue samples and cell lines were tested by immunohistochemistry and immunofluorescence staining, mRNA were quantified by qRT-PCR technique (including TaqMan technology). Two specific H_4R agonists ST-1006 and HST-10 were used for *in vitro* functional (HSG), internalization and apoptosis (NS-SV-AC) cell line experiments. Levels of IL-8 and VEGF produced by HSG cells ± ST-1006 were analyzed by xMAP and ELISA assays. [^{3}H]histamine transport in HSG cells ± OCT3 inhibitor MMP was analyzed by liquid scintillation technique. hrTNFα/IMD0354-indiced apoptosis of NS-SV-AC cells ± HST-10 was analyzed with following techniques: Western blot to study late apoptosis marker cPARP, anti- and pro-apoptotic proteins BAX and Bcl-XL and phosphorylation of JNK and ERK MAPKs; flow cytometry to study Annexin-V and PI labeling of apoptotic and necrotic cells; phase-contrast microscopy to study morphological changes of apoptotic cells; additionally, BAX and Bcl-XL mRNA levels were studied by qRT-PCR.

Results. H_4R was found in the acinar and ductal epithelium in healthy salivary glands on both mRNA and protein levels. Immunohistochemical staining showed relatively low H4R expression in samples from SS patients as compared to those from healthy controls. Healthy ductal salivary epithelium is fully equipped with a histamine synthesizing, transporting and intracellular degrading machinery. SS salivary glands were characterized by drastically diminished expression of major histamine transporter OCT3 at both protein and mRNA levels. *In vitro* experiments showed time dependent up-regulated production of IL-8 and VEGF by HSG cell upon high-dose stimulation of H_4R with ST-1006 agonist. NS-SV-AC cells express H_4R, which internalization was delayed by clathrin inhibitor methyl-β-dextrin. qRT-PCR and [^{3}H]histamine transport experiments proposed OCT3 as the major histamine transporter in HSG cells. hrTNFα/IMD0354-induced apoptosis of NS-SV-AC cells was successfully inhibited by HST-10-induced activation of H_4R in a dose-dependent manner via inhibition of JNK MAPK pathways and up-regulation of Bcl-XL anti-apoptotic protein. mRNA levels of salivary moH_4R relatively low in orhydectomized HDC^{+}/HDC^{+} as compared to intact control.

Conclusions. Salivary ductal epithelial cells are equipped with a HDC/OCT3/HNMT-machinery therefore considering them as non-professional histamine-producing cells. H_4R-mediated locally produced low nanomolar levels of histamine maintain homeostasis of the salivary epithelium. H_4R activation favors cell survival. Altered histamine transport together with decreased expression of H_4R in salivary epithelium can contribute to SS pathogenesis by predisposing epithelial cells to an apoptotic pre-condition. Mast-cell-derived high concentrations of histamine in SS salivary glands may excessively stimulate H_4R, leading to an up-regulated production of pro-inflammatory IL-8 and VEGF, followed by a down-regulation of H_4R. Diminished expression of H_4R in salivary epithelium in SS patients may also be aggravated by local/systemic androgen levels. In future, local use of low molecular weight H_4R agonists may become an alternative for costly biologicals in the treatment of autoimmune diseases, including Sjögrens' syndrome.

4. REVIEW OF THE LITERATURE

4.1. Salivary glands

The salivary gland is an exocrine gland, with one or more excretory ducts, which open into the oral cavity. In humans three major salivary glands exist comprising paired parotid, submandibular, and sublingual glands. The more superficial minor salivary glands are located in different areas of the oral cavity in the submucosa and are named based on their location to e.g. lingual, labial, buccal, molar and palatine glands.

4.1.1. Histological structure

The secretory acinus is a terminal segment of any salivary gland. According to the cellular profile, acinus can be serous (protein-secreting), mucous (mucin-secreting) or mixed (containing both types of the cells). Some mucous acini have a cap of serous cells that secrete into the highly convoluted intercellular space between the mucous cells (Figure 1). It is assumed that such visually observed phenomena of serous demilunes (caps) are a result of artificial dehydration of the serous cells during

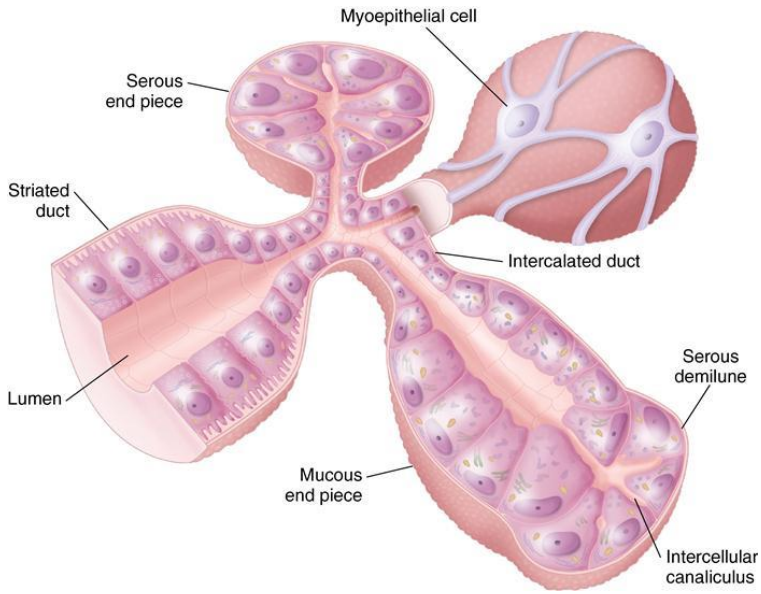


Figure 1. Schematic diagram of the salivon – a functional unit of the salivary gland.
(*Modified from Nanci A. In Ten Cate's Oral Histology: Development, Structure and Function 7th edition, 2007, Elsevier Health Sciences*)

fixative treatment. In humans, distribution of certain acini varies between the glands. For instance, the submandibular gland is a mixed gland consisting mainly of serous acini, the sublingual gland is mostly mucous, while the parotid gland is totally serous. The minor salivary glands are mucous. The relative frequency of these three types of acini is a feature by which the salivary glands can be distinguished.

The luminal spaces of the salivary acini open up to a ductal system, which consists of three sequential segments: intercalated duct, striated duct and interlobular/excretory duct. Depending on the nature of the acinar secretion, the ductal system may exhibit differently developed segments. Serous glands have well-developed intercalated and striated ducts to modify the composition of the final saliva by absorption and additional secretion. In turn, mucous glands have poorly developed segments that are to a lesser degree responsible for saliva modification, but simply present a ramified outlet system for saliva. Together with the secretory acini, these consecutive ducts form the main functional unit of the gland – salivon.

The salivary acini are embraced by contractile myoepithelial cells, which also underlay the epithelium of the proximal portion of the duct system. Myoepithelial cells lie between the basal plasma membrane of the epithelial cells and basal lamina (basement membrane). These cells actively contribute to movement of the secretory products towards the excretory duct and therefore to the oral cavity. Basal lamina consists mainly of collagen type IV and laminins combined by nidogen-1 and perlecan (LeBleu *et al.* 2007). Basal lamina participates in the differentiation and maturation of the acinar cells through an outside-in signalling mechanism. Certain chains of laminin molecules correspond to specific epithelial cells and participate in the communication between basal lamina and underlying epithelial cells. For example, laminin α1-chain is mainly connected with the acinar cells, whereas α2-chain corresponds to the myoepithelial cells (Strassburger *et al.* 1998; Laine *et al.* 2008).

4.1.2. Saliva

Mixed saliva in the oral cavity is a mucoserous substance produced mostly by the salivary glands and to a smaller extent by the gingival sulcus, tonsillar crypts, and general transudation from the epithelial lining of the oral cavity. Contribution of different salivary glands to the total daily volume of saliva is divided as follows: 65% from submandibular gland, 20% from parotid, 7-8% from sublingual, and about 10%

from numerous minor salivary glands. Interestingly, that part from the minor salivary glands is very important due to resting salivary flow, which protects oral mucosal membrane all over the oral cavity from drying between the meals. The average daily saliva volume varies from 1 to 1.5 L, which exceeds other digestive secretions by as much as 40 times measured per weight of glandular tissue (Ross *et al.* 1995; Humphrey and Williamson 2001).

Regulation of secretion

Saliva secretion and release are regulated by both sympathetic and parasympathetic nervous fibers of the autonomic nervous system. Excitation of the sympathetic fibers in the T1-T3 region transmitted forward via sympathetic trunk results in an increase of mucin-rich saliva from mucous cells. Dramatically increased sympathetic stimulation reduces salivary flow leading to dry mouth. Parasympathetic innervation via the VII cranial facial nerve of the sublingual and submandibular, and via the IX cranial glossopharyngeal nerve of the parotid gland causes enhanced serous water-rich saliva production upon food ingestion (Marieb and Hoehn 2005).

Formation and composition of saliva

Primary saliva is formed upon cholinergic stimulus from the postganglionic parasympathetic nerve fibers, leading to intracellular Ca^{2+} increase. In turn, Ca^{2+} activates basolateral and luminal (apical) membrane channels, which allow escape of K^+ and Cl^- out of the cell into interstitial space and lumen, respectively. This results in cell shrinkage and concomitant outward flow of intracellular HCO_3^- after Cl^-. Loss in HCO_3^- activates carbonic anhydrase mediating a conversion of CO_2 and H_2O into HCO_3^- and H^+. *De novo* synthesized HCO_3^- anions continue to flow outwards and the cell undergoes acidification, which in concert with the negative potential of the acinar lumen draws interstitial osmotically active sodium cations (Na^+) into the lumen via a paracellular route. Once intracellular Ca^{2+} decreases, Na^+-H^+-exchanger is activated and H^+ cations are excreted outside the cell. Na^+-coupled K^+-$2Cl^-$-inward co-transporter brings osmotically active Na^+ back to the cell, which again swells and gets smoothed. Na^+-K^+ pump (Na^+/K^+-ATPse) re-establishes the high intracellular potassium concentration and Cl^--HCO_3^--exchanger restores intracellular HCO_3^- and pH. Contraction of the myoepithelial cells forces saliva out of the acinar lumen into the intercalated duct. Later, passing striated and excretory ducts, saliva undergoes

ionic modification. The ductal epithelium reabsorbs Na^+ and Cl^-, and secrete K^+ and HCO_3^- (Nauntofte 1992). Apart from 95-99% water component, composition of saliva includes various ions, hormones, enzymes, immunoglobulins and cytokines (Humphrey and Williamson 2001). Each compound has its own function. Salivary histamine at resting phase varies within 3–112 nM (0.3–12 ng/ml) concentration levels (Kejr *et al.* 2010).

Functions of saliva

Among the many functions of saliva, lubrication and moistening of the oral mucosa could be mentioned first. Most important components of saliva, which coat the oral cavity, are water-binding gel-like mucins that are excreted from minor salivary glands. These complex glycocylated protein molecules (glycoconjugates) have low solubility, high elasticity, high viscosity, and strong adhesiveness. By lubrication and moistening they facilitate mastication, speech and swallowing, and also provide a molecular mechanical barrier against chemical irritants and harmful bacteria. Mucins have been shown to participate in the regulation of intercellular calcium levels and to initiate bacterial colonization of the oral cavity by benign and probably mucoprotective commensal flora (Denny and Denny 2008).

Saliva also provides immune defence mechanisms for the oral cavity, which include systemically and locally produced immunoglobulins, lysozyme and a wide spectrum of antimicrobial peptides (AMP). IgA was identified to be the dominant form of immunoglobulins immobilizing salivary bacteria. The cationic enzyme lysozyme causes damage to the Gram-positive bacterial cell wall, therefore being able to protect oral epithelium from foreign bacteria containing peptidoglycan cell wall (Schenkels *et al.* 1995). As part of a pool of antimicrobial agents, antimicrobial peptides have been shown to play an important role in a cell-free immune response in the oral cavity (Hancock and Lehrer 1998). The most prominent representatives of AMPs in the mouth are cathelicidin LL-37, defensins and lactoferrin. AMP LL-37 is a multifunctional molecule capable of killing pathogens and modulating immune response, as well as stimulating mast cell histamine release. In turn, defensins have been shown to strengthen the antimicrobial activity of cathelicidines, augmenting the membrane permeabilization of target cells (Niyonsaba *et al.* 2001; Scott *et al.* 2002; Yang *et al.* 2002). Oral lactoferrin acts as an iron scavenger. The studies show that deprivation of iron prevents formation of pathogenic bacterial biofilms (Singh 2004).

Oral epithelium is known to be one of the fastest regenerating tissues in the human body. This repair acceleration is reached in presence of epidermal growth factor (EGF) in the oral mucosa and saliva (Wang *et al.* 1990). Along with antimicrobial properties, histidine-rich proteins histatins, have also been proposed to be involved in oral tissue repair (Xu *et al.* 1991; Oudhoff *et al.* 2008).

Salivary amylase and lipase are involved in food digestion, degrading starch and lipids already in the oral cavity. Bicarbonates, phosphates, urea and carbonic anhydrases regulate the buffering capacity of saliva and protect against acid attack. Cystatins and statherins are involved in bone and teeth mineralization processes (Johnsson *et al.* 1991; Humphrey and Williamson 2001; de Sousa-Pereira *et al.* 2013).

Factors affecting saliva production

Hypersalivation can be caused by number of reasons and conditions, including various neuronal disorders, Parkinson's disease, gastro-esophageal reflux disease and hyperhydration. Increased saliva production can be detected also in the case of heavy metal poisoning, or triggered by medicines stimulating post-ganglionic parasympathetic nerve fibers, such as pilocarpine, bethanechol, lithium etc., as a drug or side effect (Smith and Burtner 1994).

Hyposalivation, in turn, can also be as result of medication, mainly of drugs that directly or indirectly interfere with the actions of acethylcholine, such as anticholinergic drugs, antihistamines, tranquilizers, sedatives, and beta-blockers. A number of systemic diseases lead to xerostomia (dry mouth). Among them (primary or secondary) Sjögren's syndrome (SS), which is an autoimmune disease, takes an essential place (Farnaud *et al.* 2010).

4.2. Sjögren's Syndrome

Sjögren's syndrome is a chronic autoimmune disease, an autoimmune epithelitis, that primarily affects exocrine glands, resulting in their functional and finally structural impairment. This leads mainly to symptoms of oral, ocular and cutaneous dryness. The syndrome was named after the Swedish ophthalmologist Henrik Sjögren, who summarized knowledge on keratoconjunctivitis sicca ("dry eyes") and provided the basis for future definition of the syndrome (Sjögren 1933). The prevalence of primary SS (pSS) is estimated to be between 0.5 and 1% (up to 5%

according to some studies and definitions) (Jacobsson *et al.* 1989; Dafni *et al.* 1997; Pillemer *et al.* 2001; Bowman *et al.* 2004; Haugen *et al.* 2008) within the population, mainly affecting middle-aged women, with a female-to-male ratio reaching 9:1, sometimes even 14:1 (Garcia-Carrasco *et al.* 2002; Mavragani and Moutsopoulos 2010). Secondary SS (sSS) manifests in 4-31% patients with rheumatoid arthritis, in 9-19% patients with SLE and in 14-20% patients with systemic sclerosis (Ramos-Casals *et al.* 2007).

4.2.1. Clinical features and classification criteria

Typical SS patient describes dry eyes (xerophthalmia) and dry mouth (xerostomia) symptoms, complaining of "gritty" or "sandy" feeling in their eyes and difficulties in swallowing dry food. Some patients regularly moisturize their oral cavity with small sips of fluids, in some cases also at night. Impaired tear fluid production, resulting in insufficient tear film formation, is often complicated with corneal ulceration and infection of the conjunctiva and Mebomian glands of the eyelids. Inadequate salivary flow may contribute to dental caries and oral candidiasis due to loss of the protective and antimicrobial properties of saliva. Among exocrine symptoms parotid swelling and other xeroses, such as dryness of skin and mucosa in the nose, throat or vagina, are frequent due to failure of the function of exocrine glands (Tincani *et al.* 2013).

Cutaneous involvement can be found in up to 50% of SS patients. Most prominently skin xerosis affects dorsal and lateral surfaces of the extremities (Bernacchi *et al.* 2005). Other skin-related symptoms in SS patients are Raynaud's phenomenon with prevalence of about 30% (Tektonidou *et al.* 1999), cutaneous vasculitis with a prevalence of about 10%, appearing as purpura, urticarial lesions, papules, small ulcers (Ramos-Casals *et al.* 2004) and annular erythemas (Kuhn *et al.* 2000).

The respiratory system also suffers in SS patients. Due to xerotrachea and laryngitis sicca patients may complain of persistent hoarseness and a chronic, non-productive cough. In about 20% of SS cases recurrent bronchial and pulmonary infections and otitis media can take place (Mialon *et al.* 1997). Increased risk of developing pulmonary extranodal marginal zone B-cell MALT lymphoma with a prevalence of 1-2% have been reported (Cain *et al.* 1998).

Non-exocrine manifestations of SS comprise a heterogeneous group of symptoms and disorders also providing evidence of systemic nature of the disease and may be

based on lymphocyte recirculation, immune complexes or other disease-associated pathomechanisms.

There are several neurological manifestations in SS, both peripheral and central. About 20 to 60% of SS patients present peripheral neuropathies, which include small fiber neuropathies, sensorymotor or pure sensory (dorsal ganglion) neuropathies, multiple mononeuropathy (mononeuritis multiplex), polyradiculopathy and autonomic neuropathy. The neurological symptoms often precede the diagnosis of SS, especially in elderly people (Delalande *et al.* 2004; Chai and Logigian 2010). Central nervous system involvement occurs rarely and can lead to diffuse or focal lesions, such as subacute aseptic meningitis, chorea, optic neuritis, transverse myelitis etc., but the prevalence of those (in range of 8-40%) is difficult to estimate because of use of different diagnostic criteria and selection bias. Many patients complain of changes in cognitive functions, poor memory and concentration abilities loss (Hietaharju *et al.* 1993; Massara *et al.* 2010).

SS patients also suffer from gastrointestinal manifestations, caused by motility disorders leading to reduced esophageal lower sphincter pressure and disturbed peristalsis. Nutcracker esophagus and achalasia have also been reported to be associated with SS (Ebert 2012). Dyspepsia, nausea and epigastric pain are complaints that SS patients can present. SS can be also associated with primary biliary cirrhosis, which is an autoimmune disease characterized by production of antimitochondrial and anti-nuclear autoantibodies and clinical cholestatis (Fox 2005; Selmi *et al.* 2012).

Prevalence of disabling fatigue among SS patients is estimated to be up to 70%. Fatigue is often connected to sleep disturbances, muscle pain stiffness and weakness. About 27% of the patients fulfil diagnostic criteria for fibromyalgia (Lindvall *et al.* 2002). Typical SS patient experiences reduced quality of life and scant social activities in advanced stage of the disease due to disturbing systemic symptoms, high-level anxiety and depression (Meijer *et al.* 2009).

Current classification criteria of SS were developed and revised several times since 1989 by an American-European consensus group (AECG) for SS. The latest most widely accepted revision was done in 2002 (Vitali *et al.* 2002). Inclusion criteria combine subjective symptoms of dry eyes and dry mouth with objective ocular and salivary signs based on tear- and saliva-flow tests, objective histopathological and serological data (Table 1). Simplified criteria have also been suggested and consist of

only 2 out of three features, autoantibodies, pathological ocular staining score and focal sialadenitis (Shiboski et al. 2012).

Table 1. Inclusion criteria for SS diagnosis (modified from Vitali *et al*. 2002)

Items	**Definition**
I. Ocular symptoms	Counts if at **least one** of the following is fulfilled: a. Daily, persistent troublesome dry eyes for more than 3 months. b. Recurrent feelings of sand or gravel in the eyes. c. Use of tear substitute more than 3 times a day.
II. Oral symptoms	Counts if at **least one** of the following is fulfilled: a. Daily feeling of dry mouth for more than 3 months. b. Recurrently or persistently swollen salivary glands as an adult. c. Frequent drinking of liquids to aid in swallowing dry food.
III. Ocular signs	Counts if at **least one** of the following is fulfilled: a. Schirmer's *I* test ≤ 5 mm in 5 min without anesthesia b. Rose bengal score ≥ 4 according to van Bijsterveld's system
IV. Histopathology (in minor SG)	Counts as focal lymphocytic sialoadenitis if focus score ≥ 1, defined as number of lymphatic foci per 4 mm^2 of glandular tissue.
V. Salivary gland involvement	Counts if at **least one** of the following is fulfilled: a. Unstimulated whole salivary flow ≤ 1,5 ml in 15 min b. Diffuse sialectasis in parotid sialography without evidence of major ducts obstruction. c. Delayed uptake, reduced concentration and/or delayed excretion of tracer in salivary scintigraphy
VI. Serology	Counts if anti-SSA/Ro or/and anti-SSB/La autoantibodies are present in the serum

Diagnosis of pSS can be approved if **(1)** any 4 of the 6 items are positive, as long as either item IV (Histopathology) or VI (Serology) is positive, OR **(2)** any 3 of the 4 objective criteria items III-VI are positive, OR **(3)** the classification tree procedure represents a valid method for classification, although it should be more widely used in clinical-epidemiological survey.

Diagnosis of sSS can be approved if in association with another well-defined connective tissue disease, the presence of items I or II is supplemented with any 2 from among items III, IV and V.

SS diagnosis should be disapproved if patient suspected for SS meets one of the following exclusion criteria: past head and neck radiation treatment, hepatitis C virus infection, acquired immune deficiency syndrome (AIDS), pre-existing lymphoma, sarcoidosis, graft versus host disease, use of anticholinergic drugs (since a time shorter than fourfold the half-life of the drug).

Considering complexity of SS diagnosis many physicians misguidedly treat each symptom individually, unaware of the systemic nature of the disease. As usual, early diagnosis can provide adequate treatment and improve patient's wellbeing (Kassan and Moutsopoulos 2004).

4.2.2. Current view on aetiology and pathogenesis

The ultimate cause of SS is not certain, however, presence of a number of signs (including biosignatures) of pathological processes discovered in the patients let us consider SS as a multifactorial autoimmune disease. Several approaches for SS pathogenesis have been proposed so far. The diseased glands are characterized by focal lymphocytic infiltration, consisting predominantly of $CD4^+$ T-lymphocytes, B-lymphocytes and dendritic cells. Histologically the salivary gland in advanced SS represents a picture of acinar atrophy associated with ductal hyperplasia. It is assumed that ductal epithelial proliferation is a response to the acinar involution process, which is due to apoptosis (Voulgarelis and Tzioufas 2010). There are increased levels of T_h1-response-related proinflammatory cytokines (IL-1β, IL-2, IL-6, IFNγ and TNFα) and T_h2-response-related antibody-stimulating cytokines (IL-4, IL-5 and IL-13) in saliva and serum of SS patients (Roescher *et al.* 2009). Circulating levels of anti-SSA/Ro and anti-SSB/La autoantibodies (one of the crucial diagnostic features) as well as rheumatoid factor, type-2 cryoglobulins and hypergammaglobulinemia are signs of high B-lymphocyte activity in SS (Kallenberg *et al.* 2011).

IFNα/β (IFN type I) signature in SS salivary glands, associated with increased numbers of IFN-producing plasmacytoid dendritic cells, gives evidence of inductive involvement of innate immunity into pathological process of the disease (Ogawa *et al.* 2002). This is in line with the up-regulation of IFN type I inducible genes, such as B-lymphocyte activating factor (BAFF) in SS (Nossent *et al.* 2008; Lahiri *et al.* 2014).

Various viral infections have been introduced as potential etiological factors for SS, because of shared features with the syndrome. However, due to similar manifestations, for instance in HCV infection, certain infections are considered, as mentioned above, as exclusion criteria for SS diagnosis. The rest, for example Epstein-Barr virus, cytomegalovirus, herpes virus-6 and human endogenous retroviruses have been proposed to be involved in SS pathological process (Ramos-Casals *et al.* 2008; Vitali 2011).

Hormonal imbalance in SS patients is proposed to be one of the contributing elements in the disease. Female dominance among the patients and the age of onset of the disease define androgen and estrogen deprivation as one of the key factors towards SS development. According to some studies, low serum levels of DHEA-S together with local dysfunction of DHEA processing steroidogenic enzymatic machinery in the diseased salivary glands, could lead to local androgen deficiency in female SS patients (Spaan *et al.* 2009). However, DHEA substitution treatment for severe fatigue pSS patients in placebo-controlled studies did not have any significant healing effect, proposing dysfunction of local androgen synthesis too (Hartkamp *et al.* 2008; Virkki *et al.* 2010). Men, in contrary, *a priori* have high enough testicle-derived systemic levels of androgens, allowing to feed the salivary glands with testosterone, which can be sufficiently effectively converted to dihydrotestosterone (DHT), which could prevent acinar atrophy and ductal cell hyperplasia caused by local androgen deficiency (Porola *et al.* 2010).

Oxidative stress along with accumulation of various molecular degradation products during aging could contribute to the loss of tolerance and to SS progression. Among them dysfunctional Toll-like receptor (TLR), T-cell receptor and B-cell receptor signalling as well as age-related changes in dendritic cells and memory B-cells (Vallejo 2011) are closely intertwined with our current knowledge of SS.

Altogether these factors in genetically predisposed people can trigger the disease and contribute to its progression. Core of the disease is a process of autoantigen presentation. It has been suggested that cryptic epitopes of Ro and La ribonucleoprotein particles are presented to plasmacytoid dendritic cells complexed with adjuvant single-stranded hY-RNA, perhaps via various membrane particles (Fabini *et al.* 2000). Once autoantigen presentation takes place and T-cells are polarized and B-cells activated, lymphocytic invasion, resulting in chronic inflammatory infiltrates and production of autoantibodies by B-cells, follow.

It has been reported that normal apical localization of aquaporin 5 is dislocated to basolateral membrane of the acinar cells in SS. Therefore, part of the water destined to secretion "leaks" into the interstitium of the salivary gland (Steinfeld *et al.* 2002). Similar phenomenon was also observed in NOD mice, a rodent model of SS (Konttinen *et al.* 2005).

The etiology and pathogenesis of SS still appears to be a subject for studies and detailed analysis. To date, there is no strait and elegant explanation for the disease but consensus states that SS is rather a multifactorial and complex autoimmune pathological entity.

Table 2. Factors proposed to play role in SS etiopathogenesis*

Factor	Mediators
Cells	T_h1, T_h17, NK-cells, B-cell, plasma cells, plasmacytoid dendritic cells, macrophages, epithelial cells, DN T-cells, T_h follicular cells
Cytokines	INFα/β, INFγ, TNFα, BAFF, VEGF, IL-1β, IL-5, IL-6, IL-7, IL-8, IL-12, IL-13, IL-17, IL-18, IL-21, CXCL13, CXCL21
Viruses	HIV, Epstein-Barr virus, cytomegalovirus, herpes virus-6 human T-cell leukemia virus, human endogenous retroviruses
Genetic factors	Polymorphism HLA-II expression (Gottenberg *et al.* 2003)
Epigenetics	microRNAs, altered methylation
Autoantibodies	SSA/Ro, SS-B/La, α-fodrin, M3 receptor (Bacman *et al.* 1996; Ulbricht *et al.* 2003)
Aging	Oxidative stress, defective signalling of T-cell receptor (TCR), B-cell receptor (BCR) and Toll-like receptor (TLR), Adrenopause with a local sex steroid processing dysfunction

*(Mavragani and Moutsopoulos 2014)

4.2.3. Treatment

Today, management of SS is a challenge for every physician. It is based on symptomatic treatment of glandular manifestations and on systemic treatment of organ involvement, damage and loss of their function (extraglandular manifestations).

Symptomatic treatment generally includes measures against sicca symptoms. Dry eye topical treatment is based on substitution of tears with artificial hypotonic solutions and emulsions that decrease tear film osmolality. Patients are advised to use eye drops 3-6 times a day and ophthalmic gels at night. In case of severe dryness, topical NSAIDs can be administered for a short time to relieve ocular pain. Glucocorticoids, such as dexamethasone drops, can be used to suppress local inflammation, but only in severe cases if adviced by a ophtalmologist. Recently, ciclosporin A (an immunosuppressive drug) has been reported to be effective in topical application for dry eye symptoms and low tear production (Tincani *et al.* 2013). There are numerous saliva substitutes available in forms of lubrication gels, mouthwashes and sprays for dry mouth relief. Frequent sips of water during mastication, use of sugar-free candies, gums or mints are also recommended for SS patients.

Medications mentioned above should be combined with a preventive non-pharmacological approach, which is based on avoidance of dry, smoky and windy environments, prolonged computer work or reading combined with the use of humidifiers. Dryness aggravating drugs such as diuretics, beta-blockers, antihistamines and drugs with anticholinergic effects should be taken with precautions or avoided. Regular oral hygiene procedures should include 0.1% fluoride mouth rinse or application of neutral sodium fluoride gel and remineralizing solutions. Alcohol, caffeine and fermentable carbohydrates-containing beverages should be minimized. Adequate follow-up, individualized treatment and preventive measures significantly decrease occurrence of complications.

Systemic treatment includes limited numbers of different disease-modifying anti-rheumatic drugs, corticosteroids, immunosuppressive drugs and biologicals. Acetycholine receptor agonists in tablets, such as pilocarpine and cevimeline, have been shown to be beneficial in the treatment of dry eyes (Mavragani *et al.* 2006). However, use of these pharmacological agents is limited, especially in elderly patients, due to side effects including sweating, flushing gastrointestinal discomfort,

urinary retention and also because of incompatibility with other diseases such as uncontrolled asthma, narrow-angle glaucoma, acute iritis and cardiovascular diseases.

Musculoskeletal pain and constitutional symptoms in SS patients can be reduced by use of NSAIDs. Antimalarial drug hydroxychloroquine has been reported to be helpful in patients with systemic features, especially arthritis and skin involvement (Kruize *et al.* 1993; Dawson *et al.* 2005). Hydroxychloroquine is non-applicable to patients with renal failure and retinal damage (Rigaudiere *et al.* 2004). SS patients can be treated with low-dose prednisone eqvivalent up to 10 mg/day. With first signs of necrotic or ulcerating vasculitis, the daily prednisone dose should be increased up to 30 mg/day. In case of severe manifestations like life-threatening central nervous system or progressive renal failure, high-dose corticosteroids (1 mg/kg daily) in combination with cyclophosphamide can be administered (de Seze *et al.* 2006).

Many pharmacological reagents that initially looked promising in SS trials have shown very modest efficacy or had no healing effect at all. Antimetabolite methotrexate showed improved subjective parameters, but no improvement of objective signs in patients with sSS and additionally had adverse effects (Group 1995). Despite positive symptomatic treatment results in RA, high prevalence of adverse effects and inefficiency in SS have been reported for azathioprine and also sulfasalazine (Price *et al.* 1998; Thanou-Stavraki and James 2008). IFNα therapy in randomized controlled trials showed a controversial outcome. There was no significant effect on oral dryness or stimulated whole salivary flow, however, non-stimulated salivary flow increased in SS patients (Cummins *et al.* 2003; Shiozawa and Shiozawa 2006). On the contrary, recently finished anti-IFNα therapy clinical trials with immunogenic interferon-α-kinoid in patients with SLE showed significant down-regulation of IFN signature (Lauwerys *et al.* 2013). Several biologicals targeting TNF-α, including infliximab and etanercept, have been reported to be ineffective in trials with SS patients (Mariette *et al.* 2004; Zandbelt *et al.* 2004). Rituximab has been reported to be helpful in SS. Administration of this chimeric monoclonal antibody specific for CD20 B-cell surface molecule has been tentatively shown to improve both subjective and objective symptoms at least for 6-9 months in patients with active pSS in randomized controlled trials and in open-label studies (Devauchelle-Pensec *et al.* 2007; Dass *et al.* 2008; Pijpe *et al.* 2009; Meijer *et al.* 2010), but it did not improve salivary flow in patients with no or almost no residual salivary flow at baseline.

Open-label study has shown promising results for epratuzumab, a humanized monoclonal antibody, which is specific for CD22 B-cell surface protein. Use of the drug resulted in improvement of objective signs and in visual analogue scale scores for fatigue (Steinfeld *et al.* 2006).

DHEA was proposed as a potential drug for SS patients, because of female dominance. However, as mentioned above, it had no healing effect in the controlled trials.

SS patients are vulnerable to oral candidiasis with a prevalence up to 30-70%, especially after treatment with corticosteroids and antibiotics. Depending on localization, topical or systemic antifungal drugs are usually administered (Soto-Rojas and Kraus 2002).

So far, symptomatic approach still remains fist line treatment for SS patients, due to lack of evidence on aetiology and complete knowledge on the pathogenesis of SS. Recent trials have shown some tentative beneficial effects of biologicals targeting B-lymphocyte-mediated and IFN type I-mediated responses. Other potential targets in pathogenetic-directed treatment include BAFF and cytokines like IL-6, adhesion molecules and chemokines (Moerman *et al.* 2013).

4.3. Histamine metabolism and transport

4.3.1. Histamine producing cells and “on/off-state” conception

Histamine is a biogenic amine, perhaps one of the most intensively studied inflammatory mediators, which participates in many pathological events, including processes in allergy and autoimmune diseases. Although histamine-producing cells vary considerably in origin and functions, they can be classified under two types, professional and non-professional histamine producing cells. Professional histamine producing cells synthesize rapidly, store in granules and release in bursts histamine into the extracellular milieu so that local histamine reaches high micromolar concentrations. Unlike professional, non-professional histamine producing cells synthesize histamine continuously at low speed, do not store it in granules and transfer it between the intracellular and extracellular space along its concentration gradient via “equilibrative uniporter” channels. This leads to a rather low local nanomolar histamine concentration. Such a dichotomy of histamine secretory cells is

connected to a similar dichotomy in the histamine affinity of histamine receptors and their involvement in certain pathological/physiological events (Konttinen *et al.* 2013). In general, low-affinity conventional H_1R and H_2R are maximally active in allergy and helminthic infections, leading to usually rather acute and transient conditions which may suffice to eliminate most of the allergen and parasitic irritants. From this point of view, it is natural that professional the histamine producing cell group comprises mobile basophils and tissue resident mast cells throughout the body, which can be recruited and activated upon need, resulting in their degranulation. Enterochromaffin-like cells (ECL) of the gastric mucosa, which take a prominent part in food digestion via activation of H_2R, can be included in the professional histamine producing cell group. In turn, high-affinity histamine receptors, in particular the novel H_4R, seem to maintain and regulate subtle underlying mechanisms responsible for homeostasis and tolerogenic immunity. Accordingly, the non-professional histamine producing cell group includes many cells involved in immune responses, namely dendritic cells, T-cells and monocytes/macrophages (Kubo and Nakano 1999; Szeberenyi *et al.* 2001; Tanaka and Ichikawa 2011). These findings suggest a novel role for histamine in immune coordinated actions, which became a hot topic last decade. In addition, non-immune cells, such as foetal liver cells, ductal epithelial cell of the salivary glands, epithelial cells of the mammary glands, skeletal muscle cells and even sperm cells produce histamine at low concentrations (Tanaka and Ichikawa

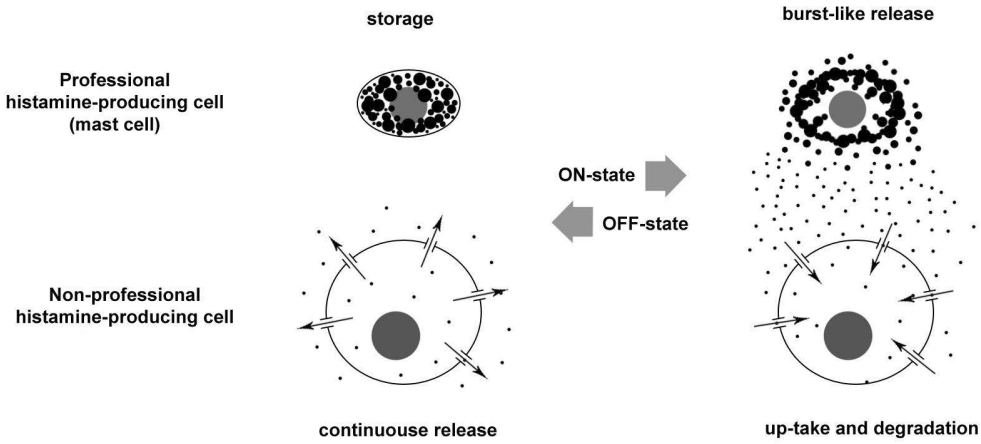


Figure 2. Histamine producing cells during off- and on-state.

2011; Niijima-Yaoita *et al.* 2012), which may help to maintain the immunological tolerance.

As well as being the main source of local nanomolar concentration of histamine during so-called "off-state" phase, non-professional histamine producing cells assume another function of being high-capacity histamine sinks, when MCs or other professional histamine-producing cells release vast amounts of histamine during short-term "on-state" phase (Figure 2). Subsequently, the intracellular histamine-N-methyl transferase (HNMT)-dependent degradation of histamine preserves cells from excessive overstimulation and brings back homeostatic histamine quantity to the milieu.

A part from a number of histamine producing cells, food, such as various fish and vegetables, is also a source of histamine (Maintz and Novak 2007). Studies show, that histidine decarboxylase (HDC) knock-out mice are not totally "histamine-free", even when kept on histamine-free diet (Ohtsu *et al.* 2001; Mondillo *et al.* 2007). In that sense, microbial intestinal flora, in particular species from *Lactobacillaceae* and *Enterobacteriaceae* families, plays a role as a minor histamine supply depot (Riley and Snell 1968; Devalia *et al.* 1989; Ohtsu *et al.* 2002).

4.3.2. Histamine synthesis, release, transport and degradation

Histamine is synthesized by histidine decarboxylase (HDC), the exclusive histamine synthesizing enzyme, which catalyses conversion of the amino acid *L*-histidine to histamine with vitamin B_6 as a cofactor (Rosenthaler *et al.* 1965) (Figure 3). The molecular mechanisms underlying professional and non-professional histamine synthesis have its explanation in the existence of two cleavage isoforms of

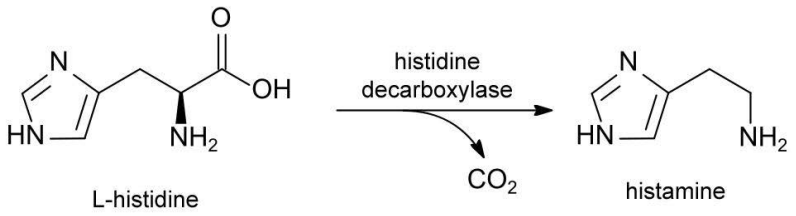


Figure 3. Histamine synthesis (simplified).

HDC: (1) low active full-length 74-kDa HDC and (2) high active cleaved 53-kDa HDC. Both of them are found in mammalian cells, but in different subcellular compartments. In non-professional histamine producing cells 74-kDa HDC is localized in the cell cytoplasm, whereas in professional histamine producing cells it is cleaved by caspase-9 in the endoplasmic reticulum (ER) and subsequently localized to the granule fraction, where it rapidly synthesizes vast amount of histamine in about 100-1000-fold higher speed than the 74-kDa HDC (Ichikawa *et al.* 2010). HDC activity varies in different conditions, like inflammation, under stimulation by cytokines, such as IL-1, IL-3, IL-12, IL-18, GM-CSF and TNFα (Jutel *et al.* 2009).

At the physiological pH values (≈7.36) histamine is a positively charged amine (monovalent cation). In non-professional histamine producing cells *de novo* synthesized histamine passes freely along concentration gradient from cytoplasm to extracellular space (or *vice versa*) via plasma membrane organic cation transporters (OCTs), OCT2 and OCT3, also known as solute carriers SLC22A2 and SLC22A3, or via plasma membrane monoamine transporter PMAT, also known as SLC29A3 and as equilibrative nucleoside transporter ENT3. These pH-dependent equilibrative uniporters do not utilize chemical ATP energy and can transport other monoamines like serotonin, dopamine and some drugs (Grundemann *et al.* 1999). This mechanism of secretion/release allows cells to achieve extracellular histamine nanomolar-, but not higher concentrations, which are only effective via H_3R and H_4R, based on their high histamine affinity reflected in their pK_i values of 8.0 and 8.2 respectively (Konttinen *et al.* 2013).

Professional histamine producing cells release histamine in a burst-like manner by exocytosis. Degranulation occurs in sensitized individuals when mast cell or basophil surface IgE is effectively cross-linked by allergen (immediate type I hypersensitivity). Such rapid release leads almost immediately to micromolar histamine concentrations, which act effectively on H_1R and H_2R (their pK_i values are 4.2 and 4.3 respectively). ECL-cells rapidly release histamine either upon direct acetylcholinergic stimulation or indirectly via D-cell-mediated somatostatin stimulation. Professional cells, like mast cells, also express OCT3, which can serve as a way for histamine recirculation into these cells. Once histamine is re-uptaken, it is further transported back to the storage granules via vesicular monoamine transporter 2 (VMAT2), also known as SLC18A2. Via VMAT2 also newly synthesized histamine passes from 53-kDa HDC into the storage granules. High amounts of histamine can be further washed away by body

fluids and is degraded extracellulary, or is taken up by non-professional histamine producing cells via already mentioned equilibrative solute carriers for further intracellular degradation. These processes are catalysed by two major histamine-degrading enzymes: diamine oxidase (DAO) and histamine-N-methyl transferase (HNMT) (Maintz and Novak 2007) (Figure 4). DAO metabolizes extracellular histamine (15-30%) to imidazole-4-acetaldehyde, which is subsequently transformed into imidazole acetate by aldehyde dehydrogenase. Studies show that DAO is released into extracellular space from vesicles located close to cell membrane upon appropriate stimulation (Maintz and Novak 2007). Mammalian DAO has been shown to be expressed mostly in small intestine, ascending colon, placenta and in renal tubular cells. HNMT appears to be the only intracellular histamine-degrading enzyme. It metabolizes the majority of histamine (50-80%) to N-methyl histamine, which is further metabolized by monoamine oxidase to M-methylimidazole, the primary urinary metabolite. HNMT is present in many epithelial cells, in particular in airway epithelium, where it is responsible for local histamine degradation released from mast cells (Barnes *et al.* 1985; Sekizawa *et al.* 1993). In mammals HNMT is also expressed in gastrointestinal tract, urinary bladder, islets of Langerhans, epidermal cells of the skin and in kidney tubules (Tahara *et al.* 2000). Altered expression or absence of DAO in the tissues together with exposure to high enough histamine may result in histamine intolerance. Impaired enzymatic activity can be due to genetic reasons or

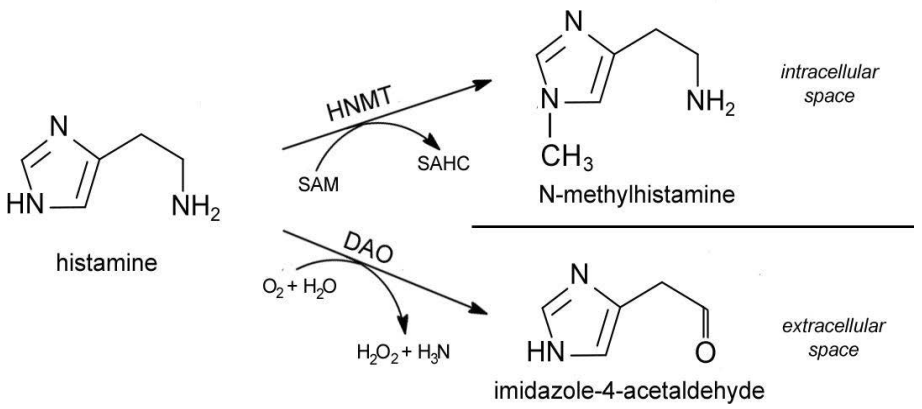


Figure 4. Histamine degradation (simplified). HNMT = histamine-N-methyl transferase, DAO = diamine oxidase. SAM = S-adenosylmethionine, SAHC = S-adenosylhomocysteine.

acquired in competitive inhibition by other biogenic amines from food (Sattler *et al.* 1988), alcohol or drugs (Sattler and Lorenz 1990; Wantke *et al.* 1994). Reduced HNMT activity is highly associated with asthma and other respiratory diseases (Nakazawa *et al.* 1994; Preuss *et al.* 1998).

4.4. Histamine receptors

For the first time multiple effects of the histamine (β-iminazolylethylamine) were described more over than a century ago (Dale and Laidlaw 1910). However, the description of the receptors came along with the first generation antihistamines (Ash and Schild 1966). Due to selective inhibition of some of the histamine-mediated effects, the existence of several types of histamine receptors was proposed. Later, the genome sequencing technologies have confirmed the empirical knowledge of different types of histamine receptors. For the time being, four types of histamine receptors have been identified. Their characteristics are briefly depicted in table 3.

Table 3. Characteristics of the histamine receptors, modified from Shahid et al., 2009

Characteristics	H_1R	H_2R	H_3R	H_4R
[a,b] Receptor described, human gene cloned	1966, 1993	1972, 1991	1983, 1999	1994, 2000
[a] Amino acids (aa),	487 aa	359 aa	445 aa*	390 aa
[a,c] Chromosomal location	3p25, 3p14-21	5, 5q35.3	20, 20q13.33	18q11.2
[d] pK_i to histamine	4.2	4.3	7.8	8.1
[a] G-protein coupling	$G\alpha_{q/11}$	$G\alpha_{s}$	$G_{i/o}$	$G_{i/o}$
[a,b] Activated intracellular signals (principal molecules)	$Ca^{2+}\uparrow$, cGMP, NF-κB, PLC↑, phospholipases A2 and D, cAMP↑, NOS	cAMP↑, $Ca^{2+}\uparrow$, protein kinase C, PLC.	$Ca^{2+}\uparrow$, MAP kinase↑, cAMP↓	$Ca^{2+}\uparrow$, MAP kinase↑, cAMP↓

cAMP = cyclic adenosine monophosphate, cGMP = cyclic guanosine monophospate, MAP = mitogen-activated protein, NF-κB = nuclear factor-κB, NOS = nitric oxide synthase, PLC = phospholipase C. *splice variants
[a](Simons 2004), [b](MacGlashan 2003), [c](Dy and Schneider 2004), d(Thurmond *et al.* 2008)

4.4.1 Histamine H_1 Receptor

H_1R is the best known histamine receptor. It is widely expressed in smooth muscle cells of airways, endothelial cells of blood vessels, central nervous system, chondrocytes, myeloid cells, such as monocytes, neutrophils, dendritic cells and also in T- and B-lymphocytes, gastrointestinal tract, heart, genitourinary system and adrenal medulla. In disease states this receptor mediates different aspects of type I allergic reaction, which includes redness, itching and swelling, so called "triple response". Allergy is a typical pathological process, which occurs in many different forms, including allergic rhinitis, atopic dermatitis, conjunctivitis, urticaria, asthma, anaphylaxis etc. H_1R has been shown to be involved in all these conditions. H_1R-mediated high levels of histamine cause bronchoconstriction, enhanced vascular permeability of capillary vessels and vasodilatation, modulation of immune-inflammatory responses via eosinophils, basophils and other already mentioned immune cells (Akdis and Blaser 2003; Togias 2003; Haas *et al.* 2008; Huang and Thurmond 2008; Simons 2008; Feng *et al.* 2013). H_1R activation causes series of various cellular responses, like expression of adhesion molecules on vascular endothelial cells, synthesis of prostacyclin, platelet-activation factor, release of von Willebrand factor and nitric oxide (NO) (Svensjo and Grega 1986; Hamilton and Sims 1987; Carter *et al.* 1988; Kishi *et al.* 1996; Li *et al.* 2003). Studies have shown that histamine can stimulate release of adrenaline and noradrenaline from adrenal medulla and induce phosphorylation of tyrosine hydroxylase via H_1R (Livett and Marley 1986; Bunn *et al.* 1995). The majority of these phenomena are due to activation of phospholipase C (PLC) upon binding histamine or other H_1R-agonist to the receptor. $G\alpha_{q/11}$–protein subunits activate PLC, which in turn generates inositol 1,4,5-trisphosphate and 1,2-diacylglycerol leading to increased cytosolic Ca^{2+} levels and activation of protein kinase C. Enhanced Ca^{2+} triggers many pathways including NO production, release of arachidonic acid from phospholipids and increased production of cAMP and cGMP (Smit *et al.* 1999). H_1R stimulates nuclear factor kappa B (NFκB) leading to production of pro-inflammatory cytokines (Leurs *et al.* 1995).

First generation of antihistamines (Mepyramine, Diphenhydramine, Doxylamine, Pheniramine, Cyclizine, etc.) has many adverse effects. Dry mouth, orthostatic hypotension, increased appetite, disturbances of circadian and cardiac rhythms and disturbed locomotive activities occurred in patients who were treated with H_1-antihistamine therapy. This was due to their non-selective effects on muscararinic, α-

adrenergic and serotonin receptors and unwanted effects in the CNS. Today, new generation of antihistamines, which cannot cross the blood-brain barrier, have been designed and taken into use (Loratadine, Ketotifen, Cetirizine). First generation compounds with sedative effects are used mostly for treatment of allergy in association with sleep disturbances and cyclical vomiting syndrome (Cyproheptadine), but also for preoperative sedation and to counteract postnarcotic nausea (Andersen *et al.* 1997; Lehmann *et al.* 2013).

4.4.2. Histamine H_2 receptor

H_2R has gained a lot of attention when its gastric localization was discovered. The receptor is expressed in parietal cells in gastric mucosa, where histamine/H_2R ligation stimulates secretion of hydrochloric acid, when histamine is released from ECL-cells localized nearby. H_2R is also expressed in many immune cells, including neutrophils, eosinophils, monocyte/macrophages, dendritic cells, T- and B-lymphocytes. In these cells H_2R often has actions opposite to those of H_1R. For example, activation of H_2R results in inhibition of T-cell proliferation, cell-mediated cytolysis and cytokine production (Melmon and Khan 1987; Hill 1990; Jutel *et al.* 2001; Gutzmer *et al.* 2005). H_2R activation diminishes eosinophil and neutrophil chemotaxis and polarizes immune responses from Th_1/M_1-type cell-mediated direction to Th_2/M_2-type by inducing DC_2s and humoral immunity (Simons 2004). H_2R is found on epithelial-, endothelial- and smooth muscle cells of blood vessels, bronchioles and bowel. It increases vascular permeability, increases production of mucus and causes bronchodilatation and relaxation of uterine muscle (Eyre and Chand 1982; Edvinsson *et al.* 1983; Ottosson *et al.* 1989; Simons 2004). It has been reported that in cardiac tissues H_2R mediates positive chronotropic (rate of contractions) and inotropic (strength of contraction) effects, whereas H_1R activation slows down heart rate (Genovese *et al.* 1988). Expression of both H_1R and H_2R in CNS has been documented and overlaps in such areas as hippocampus, thalamus, cerebral cortex, with a low expression in cerebellum. Hypothalamus is mostly H_1R positive, whereas H_2R prevails in the basal ganglia.

Signalling of H_2R is predominantly mediated by cyclic AMP-producing adenylate cyclase second messenger system via a GTP-dependent mechanism. In turn, cyclic AMP activates a multiple substrate-oriented phosphorylating enzyme, protein kinase A (PKA), which results in certain cell responses (Shahid *et al.* 2009). Studies have

shown that H_2R stimulation results in elevated intracellular Ca^{2+} levels via inositol trisphosphate (Delvalle *et al.* 1992). However, this phenomenon cannot be reproduced in H_2R-trasfected cells, suggesting that H_2R/Ca^{2+}-mediated signalling mechanism is highly cell-specific (Leurs *et al.* 1994). Activation of H_2R stimulates c-Fos, c-Jun and p70S6 kinase (Dy and Schneider 2004; Jutel *et al.* 2009).

The introduction of H_2R antagonists together with PPI led to important changes in the treatment of gastric and duodenal ulcer and gastro-esophageal reflux disease. Surgical treatments, such as gastric resections, with or without vagotomy, were successfully replaced with gastric acid production inhibiting compounds. Fortunately, these drugs have only a small number of CNS-derived adverse effects limited to vertigo, and more rarely headache based on postsynaptic expression pattern of H_2R (Haas *et al.* 2008). Inhibitory effects of H_2R antagonists, especially cimetidine, on the cytochrome P450 system in liver may cause interaction with other drugs, which are metabolized by this enzymatic machinery (Levine *et al.* 1998). Due to a competitive affinity to dihydrotestosterone (DHT) receptor, such adverse effects like galactorrhea in women and gynecomastia in men were reported for cimetidine use in a long-term treatment (Sawyer *et al.* 1981; Sabesin 1993). Later synthesized and marketed drugs (Famotidine, Lafutidine etc.) have solved the problem of exaggerated hormonal adverse effects, but elevated risks of developing food allergies and constipation or diarrhea, due to suppressed acid-mediated breakdown of proteins, have been reported (Pali-Scholl and Jensen-Jarolim 2011).

4.4.3. Histamine H_3 Receptor

The highest density of H_3R expression is observed in CNS, with highest levels in basal ganglia, cortex, hippocampus and striatal area. Involvement of histaminergic neurons in sleep-awake cycle, energy homeostasis and cognitive processes has attracted the researches to study the possible role of histamine in the pathogenesis of various mental disorders. Functional relationships between H_3R-positive mast cells and peripheral histaminergic neurons, which originate from the tuberomamillary nucleus, suggest a role for H_3R in immunomodulation (Dimitriadou *et al.* 1994; Haas and Panula 2003; Haas *et al.* 2008). A recent study has shown that presynaptically located H_3R activates negative feedback mechanisms, which result in inhibition of histamine from neurons (Teuscher *et al.* 2007). Activation of H_3R inhibits release of a number of other neurotransmitters, such as acetylcholine, serotonin, noradrenaline

and dopamine (Schlicker *et al.* 1989; Molderings *et al.* 1992; Schlicker *et al.* 1992; Schlicker *et al.* 1993). Prominent inhibitory effect of H_3R on release of tachykinins (substance P, neurokinin A, neurokinin B) and calcitonin-gene related peptide (CGRP) has been discovered in a number of peripheral tissues (Delaunois *et al.* 1995; Imamura *et al.* 1996). It was proposed that in gastric mucosa H_3R is expressed in local mast cells or ECL cells, inhibiting professional burst-like histamine release and therefore gastric acid secretion (Soldani *et al.* 1993). On the other hand, *in vitro* studies show that H_3R stimulation leads to adrenocorticotropic hormone release from a pituitary cell line (Clark *et al.* 1992). Interesting observations have been made on H_3R knock-out mice. These animals exhibited increased body weight, due to increased food intake and reduced energy consumption. It is in line with the data of up-regulated insulin and leptin resistance and down-regulated expression of energy homeostasis-assosiated genes UCP1 and UCP3 (Rouleau *et al.* 2002; Takahashi *et al.* 2002).

Mechanism of H_3R action is still the matter of scrupulous studies, but there is increasing evidence of $G_{i/o}$ protein-dependent mechanism for being responsible for the receptor action. H_3R activation leads to inhibition of cyclic AMP production, accumulation of Ca^{2+} and stimulation of mitogen-activated protein kinase (MAPK) pathways (Cherifi *et al.* 1992; Dimitriadou *et al.* 1994).

To date, there are no commercially H_3R modulators available for routine treatment. Patients with allergic disorders, mental and CNS degenerative diseases have entered phases II-III controlled clinical trials of H_3R inverse agonist. (Passani and Blandina 2011). Trials and validation of the results are still going on. For example, Pitolisant (BF2.649) has been granted orphan drug status in the EU and USA for the treatment of narcolepsy (van der Heide *et al.* 2015). Another compound, known as PF-03654746 has shown promising results in treatment of allergen-induced nasal symptoms in combination with second-generation antihistamine fexofenadine (Stokes *et al.* 2012). GSK239512 improved episodic memory in patients with mild-to-moderate Alzheimer's disease, however, no improvements were observed on executive function/working memory or other domains of cognition (Grove *et al.* 2014). Unfortunately H_3R modulatiors were not superior to placebo in the treatment of cognitive impairment in patients with schizophrenia (M *et al.* 2013; Haig *et al.* 2014) and in the treatment of adult attention-deficit/hyperactivity disorder (Herring *et al.* 2012).

4.4.4. Histamine H_4 receptor

The pharmacological discovery of H_4R was done over 20 years ago when nanomolar doses of histamine were shown to increase intracellular Ca^{2+} in eosinophils about 7.5-fold more effectively in comparison to specific H_3R agonists R-MeHA and N-MeHA (Raible *et al.* 1994). Later, several groups have cloned the H_4R protein in different cell lines (Nakamura *et al.* 2000; Oda *et al.* 2000; Liu *et al.* 2001; Nguyen *et al.* 2001; Zhu *et al.* 2001). Since then, H_4R has become a hot topic but still rather little is known of the biological function of the receptor. Localization of H_4R is relatively restricted to hematopoietic cells, in particular to eosinophils, neutrophils, basophils, mast cells, NK-cells, dendritic cells and T-lymphocytes (Damaj *et al.* 2007). This expression in both myeloid and lymphoid lineages proposes an important role in inflammation and immune responses. Functional studies show recruitment of neutrophils from bone marrow upon H_4R activation (Takeshita *et al.* 2003). H_4R-mediated diminished production and secretion of CCL2 chemokine in monocytes (Dijkstra *et al.* 2007). There are some studies reporting H_4R-mediated chemotactic activity in mast cells, eosinophils (Buckland *et al.* 2003; Hofstra *et al.* 2003; Ling *et al.* 2004) and human monocyte-derived dendritic cells along with suppression of IL-12p70, an important NK-cell activator (Gutzmer *et al.* 2005). $CD8^+$ T-killer cells show histamine/H_4R-dependent release of IL-16, a chemoattractant and T-cell modulator (Gantner *et al.* 2002).

Apart from the hematopoietic cells, low-density expression of H_4R has been shown in central and peripheral nervous systems, which however is still the matter of debates. The receptor was found in neuronal cells of nasal mucosa (Nakaya *et al.* 2004), anterior horn of the spinal grey matter and dorsal root ganglia in mice, suggesting expression in lower motor and primary sensory neurons, respectively (Kajihara *et al.* 2010; Lethbridge and Chazot 2010), in primary vestibular neurons in rat (Desmadryl *et al.* 2012), in enteric neurons in the Meissner's submucosal neural network (Breunig *et al.* 2007), somatosensory cortex in mice and other parts of CNS of rodents and humans (Zhu *et al.* 2001; Connelly *et al.* 2009; Strakhova *et al.* 2009).

There are some reports about H_4R expression in other types of cells, such as skin fibroblasts and keratinocytes, fibroblast-like type B and macrophage-like type A synovial lining cell, chondrocytes (Yamaura *et al.* 2013) and osteoclasts (Biosse-Duplan *et al.* 2009).

Studies of regulation of H_4R expression are in progress. Genomic-based approach of the human H_4R with detailed promoter region mapping has revealed several binding sites including those for ISRE, IRF-1, NF-κB and NF-IL-6. This finding predicts that H_4R receptor gene expression might be stimulated by factors such as IFN, TNF-α or IL-6 (Coge *et al.* 2001). Recent *in vitro* studies showed interferon- and micromolar histamine-dependent down-regulation of H_4R mRNA expression in oral epithelial cell (Salem *et al.* 2015). Furthermore, IL-10 and IL-13 have been shown to down-regulate H_4R expression in monocytes, T_h2 and dendritic cells (Morse *et al.* 2001). This intriguing interplay between inflammatory and anti-inflammatory factors with H_4R may have cellular phenotype-dependent implications on the immune network.

H_4R is implicated in cancer- and cell cycle-related processes. Selective activation of H_4R *in vitro* in human cancer cells of different origin suggests anti-proliferative effects, which has been demonstrated in pancreatic carcinoma cells (Cricco *et al.* 2008), breast cancer cells (Medina *et al.* 2008) and melanoma cells (Massari *et al.* 2011; Massari *et al.* 2013). This effect is apparently due to cell cycle arrest at G0/G1 and/or G1/S stages without signs of apoptosis (Petit-Bertron *et al.* 2009). Only human breast cancer cells *in vitro* and *in vivo* (heterologous injection into mouse) went into apoptosis (Medina *et al.* 2011; Martinel Lamas *et al.* 2013). The most intriguing results on H_4R activation have been reported in rodents in *in vivo* models of radiation-induced apoptosis. In all experiments 0.1 mg/kg histamine reduced apoptosis and preserved the function of mice bone marrow cells (Medina *et al.* 2010), mice submandibular salivary gland epithelium (Medina *et al.* 2011) rat uterus and both mice and rat small intestinal mucosa (Medina *et al.* 2007; Carabajal *et al.* 2012). Similar results in rats with diminished intestinal mucosal atrophy, reduced bone marrow aplasia and reduced salivation and atrophy and reduced apoptosis of cells in SMG were observed when 10 mg/kg JNJ7777120 was administered (Martinel Lamas *et al.* 2013). JNJ7777120 is a H_4R ligand with a biased signalling, exhibiting both partial agonism or reverse agonism in different biological native systems (Rosethorne and Charlton 2011; Seifert *et al.* 2011).

Being coupled to $G_{i/o}$-protein H_4R initiates various transduction pathways such as inhibition of cAMP formation, MAPK phosphorylation, in particular of ERK1/2 (Massari *et al.* 2013), enhanced calcium influx and formation of diacylglycerol (DAG) (Oda *et al.* 2000; Zhu *et al.* 2001). Although H_4R is 43% homologous with

H_3R (Morse *et al.* 2001) and their signalling characteristics are similar, their actions can overlap but also contradict each other, as shown in the case of *in vitro* H_3R-induced apoptosis (Cricco *et al.* 2008; Medina *et al.* 2008). In the cellular networks H_3R/H_4R co-actions seem to be in balance, perhaps in part due to different cellular expression profiles (Nguyen *et al.* 2001; Shi *et al.* 2012).

Since the discovery of H_4R numerous ligands were synthesized and proposed as potential drugs targeting the receptor. Some of these compounds have been tested *in vivo*. For example, the use of JNJ7777120 in animal models of peritonitis suggests a potential role for the compound in the treatment of inflammation (Thurmond *et al.* 2004).

Several compounds with a pyrimidine moiety have entered into clinical trials. Among them H_4R antagonists UR-63325 (Palau Pharma) and ZPL-38937887 (Ziarco Pharma) showed promising results in phase I studies of allergic respiratory diseases. However, JNJ39758979 (Johnson and Johnson) did not pass phase II trial and has been withdrawn due to agranulocytosis (Salcedo *et al.* 2013). Compound INCB38579 (Incyte Corp.) has shown potential therapeutic value in preclinical studies in the treatment of dermatological diseases (Shin *et al.* 2012).

4.4.5. Constitutive receptor activity

A widely accepted two-state model of receptor activation describes ligand-receptor interactions for H_4R and many other G-protein coupled receptors (GPCRs) (Leff 1995). The model predicts the existence of the receptor in two isomeric states, *resting* or ground state (R, GDP-bound) and *active* state (R*, GTP-bound). Most receptors assume the resting (non-signalling) ground state in the absence of ligand. Some receptors may in part assume the activated (signalling) state in the absence of ligand. This means that the receptor has some intrinsic constitutive activity. In the absence of ligand the distribution of the two states is determined by the *equilibrium constant*. The binding strength of the ligand to its receptor is referred to as *affinity*, whereas the effects of this binding on the receptor and its associated signalling system are referred to as *efficacy*. A ligand has affinities to both states of the receptor. If affinity of the ligand is higher to receptor's active state, ligand is called an agonist (positive efficacy), as the receptor/G protein coupling shifts the equilibrium towards R*, followed by subsequent signal transduction. If the affinity of the ligand is higher to the resting state, it is called an inverse agonist (negative efficacy), as coupling shifts

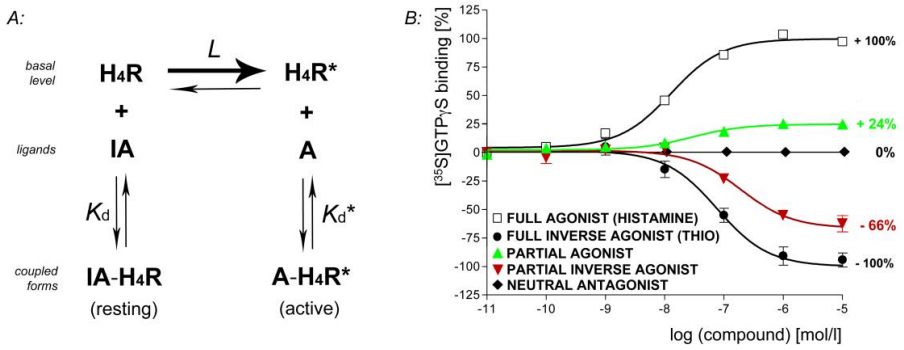


Figure 5. *A:* Two-state model of H_4R activation. IA= inverse agonist, A= agonist. K_d= dissociation constant, *L*= equilibrium constant. The model shows shifted equilibrium towards active H_4R^* form, representing high constitutive (basal) activity of the receptor. *(Modified from Leff 1995)*
B: Functional binding assay with [^{35}S]GTPγS on membrane preparation of Sf9 cells expressing hH_4R, co-expressed with Gai2 and Gb1c2 subunits. *(Modified from Sander et al. 2009, to show efficacies of different ligands)*

the equilibrium towards R and constitutive receptor activity in inhibited. If coupling does not affect the R-R* equilibrium, the ligand is called a neutral antagonist (zero efficacy) or a blocker. It possesses equal affinity to both R and R* states, so it competes with the agonists for the receptor binding sites without having any influence on response when applied alone. *Affinity* is an ability of the ligand to bind to the receptor by non-covalent intermolecular interactions. Mathematically affinity can be expressed using the receptor-ligand (AR) dissociation constant K_d=[A]×[R]/[AR]. The ratio K_d:K_d* is *efficacy* and defines the extent of the effect of the ligand. When K_d:K_d* >1, the ligand behaves as an agonist; when K_d:K_d* <1 the ligand behaves as inverse agonist; when K_d:K_d* =1, the ligand is considered a neutral antagonist (Figure 5, *A*). In case of H-receptors, histamine is a conventional physiological full agonist, whose efficacy is defined as 100%. Upon pharmacological studies of H-receptors, compound thiopyrimidine (THIO) has been considered a full inverse agonist for H_1R, whose efficacy is defined as –100%. Subsequently this definition was later applied to other discovered H-receptors, including H_4R. The efficacies of all newly synthesized potential H-receptor ligands are calculated based on the conventional reference means of histamine and thiopyrimidine (Figure 5, *B*).

Some receptors spontaneously adopt R* state and promote G-protein signalling in the absence of agonist, which is referred to as *constitutive* or basal *activity*. H_4R possesses extraordinary high constitutive activity, especially in comparison to H_1R and H_2R, constitutive activity of those is moderate and low-moderate (Seifert and

Wenzel-Seifert 2002). The studies show that the active-state H_4R^* is insensitive to high Na^+ (100 mM) concentrations, so called ionic lock, which plays role in affecting access to the binding site. H_4R also exhibits low G-protein activation kinetics, that suggests sustained and prolonged responses to histamine under physiological conditions and in long-term inflammatory processes. This is in line with report on the existence of human H_4R in a G-protein-independent high-affinity conformation without mammalian G-proteins in transfected cells. Interestedly, histamine and THIO do not affect H_4R expression, but cause conformational stabilization, unlike many other inverse agonists and neutral antagonists, which up-regulate specific receptors. Therefore it is suggested that inverse agonists refold denatured receptors, leading to increasing number of the binding sites without increasing the number of receptors on the cell membrane. In addition to this so-called "functional up-regulation", changes in glycosylation pattern, which is important for the cell surface expression of histamine receptors, is suggested to play a regulatory role for the constitutive activity (Schneider *et al.* 2009).

4.5. Histamine and salivary secretion

The first report on presence of histamine in salivary glands extracts from vertebrates and invertebrates appeared over 60 years ago (Erspamer and Boretti 1951). Later, discovery of H_1R in 1966 and H_2R in 1972 entailed new era of histamine research and first generations of antihistamines were synthesized. Before discovery of H_3R and H_4R, all findings of histamine effects on the salivary glands were considered only through H_1R and H_2R. Production of saliva comprises two simultaneously ongoing responses: salivary response, which is defined by contraction of myoepithelial cells expelling the secretions into the ductal system of the gland, and blood flow response, defined by local vasodilatation, due to vascular smooth muscle relaxation. Involvement of histamine to these responses was assumed and proposed by a number of researches (MacKay 1927; Babkin and Mackay 1931; Gibbs and McMlanahan 1937; Emmelin 1966). It was later shown that both responses are sensitive to histamine, that caused increased salivary and blood flow in dog salivary glands (Shimizu and Taira 1980). The authors reported decreased salivary response to histamine in the presence of Na^+-channel blocker tetrodotoxin and M-acetylcholine

receptors blocker (–)-hyoscyamine, suggesting that the motor effect of histamine on myoepithelial cells might be indirect via excitation of innervating parasympathetic nerves. A direct effect of histamine on myoepithelial cells was not observed, which contradicted some earlier findings (Babkin and Mackay 1931). The blood flow response, however, was resistant to any of the used M- and N-acetylcholine receptors blockers, H_2-receptor blocker (metiamide), but was sensitive to H_1-receptor antagonist mepyramine. This result suggested a direct dilatation effect of histamine on vascular smooth muscle rather than stimulation of parasympathetic postganglionic neurons. Salivary response to histamine was abolished in 15 min after mepyramine infusion. It was concluded that the leading role of histamine in the regulation of both salivary and blood flow response is H_1R-mediated. To our knowledge, since then and after the discovery of H_3R and H_4R, no reports visualizing H-receptors by immunostaining in human salivary glands have been published.

Despite the fact that ductal epithelium contains only about 10% of all salivary gland histamine (Erjavec 1985), local histamine concentration (2.8-112.7 nmol) (Kejr *et al.* 2010) is sufficient to half maximally activate highly sensitive H_3R and H_4R (pK_i H_3R = 7.8, pK_i H_4R = 8.1) (Thurmond *et al.* 2008). The rest of histamine is stored in the mast cells, the number of which positively correlates with the focus score count in the inflamed salivary glands in Sjögren's syndrome patients (Konttinen *et al.* 1990).

4.6. Apoptosis and its role in SS

Apoptosis is a physiological programmed cell death in multicellular organism. Billions of cells die by apoptosis every day in adult humans. The term *apoptosis* derives from the Greek word ἀπόπτωσις meaning "the falling-off leaves". It was introduced by Kerr with co-authors in 1972 (Kerr *et al.* 1972). This built-in cell death mechanism is essential in biological processes such as embryonic development, elimination of dangerous autoreactive T- and B-lymphocytes in the lymphatic nodes and maintenance of a ready supply of short-lived neutrophils in the bone marrow, which can be rapidly mobilized to fight infection. Cells with non-repairable damaged DNA usually kill themselves by undergoing apoptosis. In adult tissues, the apoptotic process is in a balance with cell division. Enhanced and rapid apoptosis usually leads

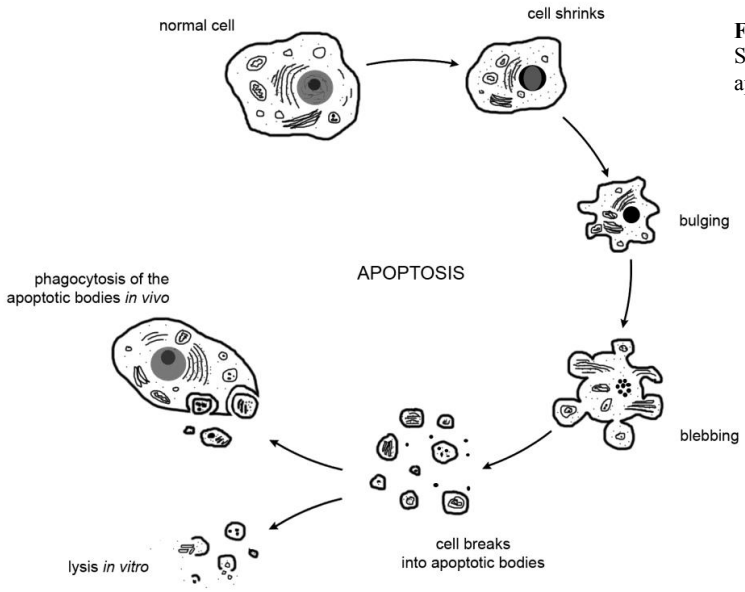


Figure 6. Stages of apoptosis.

to atrophy. Reduced apoptosis, however, may result even in cancer (Haanen and Vermes 1996; Alberts *et al.* 2008).

The cell undergoing apoptosis displays characteristic morphological changes. First, the cell shrinks and condensed chromatin collapses into crescents around the nuclear envelope. Then, the membrane begins to bulge and bleb. Finally, blebbing increases and the cell breaks into number of apoptotic bodies, which are phagocytosed *in vivo* and lysed *in vitro* (secondary necrosis) (Alberts *et al.* 2008) (Figure 6).

Cell damage inducing factors such as heat, radiation, hypoxia, increased intracellular calcium and direct cell-to-cell contacts can trigger apoptosis. Among others two major apoptotic pathways have been widely studied, namely extrinsic and intrinsic. The first one requires activation of Fas receptor (FasR), also known as apoptosis antigen 1 or cluster of determination 95 (CD95), by Fas ligand (FasL), which is expressed by cytotoxic T-lymphocytes. Activation of FasR triggers Fas associated death domain (FADD) binding, which facilitates activation of caspase-8 and therefore starts a proteolytic apoptotic cascade. Tumor necrosis factor alpha (TNFα), a proinflammatory cytokine and immune cell regulator, activates TNF receptor 1 (TNFR1 or CD120) , that triggers TNFR1-associated death domain protein (TRADD) recruitment and subsequent activation of FADD as well. However, recruitment of TRADD also initiates pro-survival NF-κB and MAPK pathways

(Inazawa *et al.* 1992; Chen and Goeddel 2002; Wajant *et al.* 2003; Lee *et al.* 2012; Liu *et al.* 2012).

The intrinsic (or mitochondrial) pathway is associated with Bcl-2 protein family members and increased permeability of the outer membrane of the mitochondrion and release of cytochrome C from the intermembrane space into the cytoplasm. Bcl-2 family comprises both pro- (BAX, Bak, Puma Noxa, Bid etc.) and anti-apoptotic (Bcl-2, Bcl-xL, Mcl-2 etc.) proteins. In non-apoptotic cells pro-apoptotic proteins are engaged with anti-apoptotic proteins and this balance secures the cell from apoptosis. Up-regulation of pro-apoptotic proteins via MAPK (JNK2, p38 etc) pathways shifts the balance and the cell undergoes apoptosis. BAX and Bak form an oligomeric pore, also known as mitochondrial apoptosis-induced channel (MAC) in the outer membrane of the mitochondrion. Released cytochrome C initiates formation of apoptosome, which in turn facilitates activation of caspase-9 and therefore starts the proteolytic apoptotic cascade (Kroemer *et al.* 2007).

The crosstalk between extrinsic and intrinsic pathways exists in Fas-mediated apoptosis. Caspase-8 cleaves pro-apoptotic BH3 interacting-domain death agonist i.e. Bid into truncated tBid form. In turn, tBid engage anti-apoptotic Bcl-2 and Bcl-xL proteins, allowing disengaged Bak and BAX to translocate to the outer mitochondrial membrane and form MAC (Wei *et al.* 2000).

Apoptosis is considered as a key event in SS pathogenesis. Salivary epithelial cells express immunoreactive molecules implicated in antigen presentation (MHC-I, II) and apoptosis (FasR). Activated epithelia also produce chemokines (CXCL13, CXCL21, BAFF) and cytokines (IL-1, IL-6, IL-8, TNF-α) contributing to creation of an inflammatory milieu and T- and B-lymphocyte infiltration. (Kong *et al.* 1997; Fox *et al.* 1999; Herrera-Esparza *et al.* 2008). The majority of infiltrating T-lymphocytes are CD4+ and their prevalence correlate with lesion severity with higher values at the intermediate stages of the disease, whereas prevalence of CD8+ autoreactive T-lymphocytes remains constant. The percentage of NK-cells, macrophages and dendritic cells is small as compared to T-cells, but it also positively correlates with lesion severity. Abundance of danger/apoptosis-inducing signals in focal lymphocytic infiltrates results in intensified epithelial apoptosis and increased production of exosomes and apoptotic bodies, containing hidden nuclear “cryptic” SS-A/B-hY RNA ribonucleoproteins and other abnormally cleaved proteins (type 3 muscarinic acetylcholine receptor, α-fodrin etc.), identified as important autoantigens in SS.

Subsequently, immunogenic autoantigens are processed by APCs, that results in B-cell driven autoimmune responses, i.e. production of autoantibodies (Nagaraju *et al.* 2001; Trokovic *et al.* 2012; Kyriakidis *et al.* 2014). Taken together, these data suggest apoptotic salivary epithelia as an initiative component of SS pathogenesis.

5. AIMS OF THE STUDY

Sjögren's syndrome is a chronic autoimmune diseases with unknown aetiology and multidirectional complicated pathogenesis. Taken together, over the last two decades continuosuly emerging data suggest salivary epithelium as a key player and conductor of autoimmune responses in the disease. Recently discovered histamine H4 receptor keeps drawing attention and interest regarding its implication on autoimmune processes. This novel receptor was found in the majority of human tissues, including immunocompetent cells and epithelium. Thus, there is a reason to believe that H_4R-mediated histamine signalling can contribute to etiopathogenesis of SS. To explore this theory the following objectives were tested:

1. The profile of human salivary gland histamine receptors and in particular expression of functional H_4R in the salivary epithelia of SS and healthy individuals.

2. Expression of histamine transporters and metabolizing enzymes in the salivary glands of SS patients.

3. The regulation of activated H_4R on Fas-mediated apoptosis of the salivary epithelium.

4. Some factors, which might explain down-regulation of H_4R expression in salivary epithelium in Sjögren's syndrome.

6. MATERIALS AND METHODS

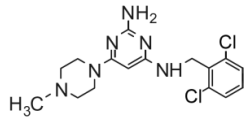
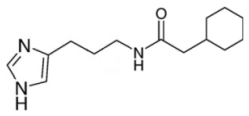
6.1. Patients and the samples (I, II, IV)

	Study I		Study II	
	SS	Healthy	SS	Healthy
Number of salivary gland samples and gender	1 man 4 women	1 man 5 women	1 man 7 women	1 man 5 women
Age, mean ± SD	48,8±5,4	47,1±5,8	53,9±6,2	47,1±5,8
Resting saliva flow, ml/15 min, mean ± SD	0,5±0,4	0,9±0,3	0,6±0,4	0,9±0,3
Focus score, mean ± SD	1,7 ± 0,3	<1	1,6±0,3	<1
Number of fulfilled SS criteria*	6 out of 8	-	6-7 out of 8	-
Fixation	IF, PCR: snap-frozen IHC: formalin, paraffin-embedded			

* Reference: (Vitali *et al.* 2002)

Snap-frozen murine salivary glands from 1-month-old BALB line (2 samples) and 4-7-months-old NOD line (7 samples) were a generous gift from our colleagues from University of Turku, Finland. Snap-frozen murine salivary glands from orchidectomized (4 samples) and non-orchidectomized (4 samples) original HDC^{+}/HDC^{+} genotype murine line, used for generation of HDC knock-out line (Ohtsu *et al.* 2001), were a generous gift from our colleagues from Semmelweis university of Budapest, Hungary. The ethics committee of the Hospital District of Helsinki and Uusimaa approved the study (19/E5/03) and all patients gave their informed consent. The study did not comprise any experiments on living animals. Murine samples were taken outside University of Helsinki according to approved by local institutions ethical guidelines.

6.2. H_4R agonists (I, III)

Code	Formula	p*Ki* hH_4R and *Ki* hH_4R, nM	E_{max}, %*	Reference
ST-1006		7,94 12 ± 3	28,4	(Sander *et al.* 2009)
HST-10		7,35 45 ± 16	85	(Kottke *et al.* 2011)

* Functional binding assay with [35S]GTPγS on membrane preparation of Sf9 cells expressing hH4R, co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ subunits. The values relate to maximal 100% response of histamine.

6.3. HSG cell culture (I, II)

The derevative from human HeLa cells (HSG), which are morphologically cervical cancer epithelial cells (earlier expected to represent human submandibular gland-derived salivary gland ductal cell line), were cultured in Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 nutrient mixture supplemented with 2-2,5 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycine and 10% fetal bovine serum (FBS, HyClone). In the study I the HSG cells were stimulated with 10 μM H_4R agonist ST-1006 for 24 hours in reduced 2% FBS medium with subsequent collection and storage at –80°C.

6.4. NS-SV-AC acinar cell culture (III)

Initially, the cells were cultured on human collagen-1 coated matrix plastic plates in K-SFM containing 100 U/ml penicillin and 100 μg/ml streptomycine, supplemented with 25 μg/ml BPE and 0,1-0,2 ng/ml rhEGF (Life Technologies) for 24 hours. Collagen-1 and BPE were necessary for attachment of the cells to the plastic surface of the plates. Next day, the medium was changed to BPE-free K-SFM, because BPE is a source of ≈42,5 nM background histamine, which is enough to compete with H_4R agonist HST-10 in further experiments. Background histamine concentration was measured with high performance liquid chromatography. Internalization and apoptosis experiments were performed 24 hours after the change of the medium.

6.5. Multiplex and ELISA assays (I)

Multiplex kit (Cat No. HCYTOMAG-60K-*PX39,* Millipore) was used to screen 39 cytokines, chemokines and other growth factors in the HSG cells medium after ST-1006 stimulation. According to manufacturer's instructions the standards and samples were pipetted in duplicates into 96-well plates and mixed with internally labelled polystyrine beads with different ratios of 2 spectrally distinct fluorophores and coated with anti-human cytokine capture antibodies for 1 hour. Later, the wells were washed 3 times and phycoerythrin-conjugated secondary detector antibodies were added for 45 min. Wells were washed twice, assay buffer was added, and samples were analyzed using a fluorescent bead–based flow cytometer equipped with 2 lasers (Bio-Plex 200 System; Bio-Rad).

As a result, two potential H_4R-regulated proteins IL-8 and VEGF were selected for subsequent validation with ELISA test system (Quantikine; R&D Systems). The analysis was also performed according to manufacturer's instructions. Pre-diluted in assay diluents samples and standards were added into 96-well plates for 2 hours and then washed 4 times with buffer. The plate surface was separately pre-coated with monoclonal antibodies against IL-8 and VEGF. Horseradish peroxidase–conjugated detector antibodies were added for 1–2 hours, followed by 3 washes and by addition of H_2O_2 substrate and tetramethylbenzidine chromogen solution for 20–30 minutes.

Absorbance at 450 nm was measured within 30 minutes using Chameleon-V microplate reader (MicroWin 2000 Lite software; Hidex, Turku, Finland).

6.6. Used Primary antibodies

Name	Immunogen	Source and clonality	Concentr. or dilution	Manufacturer or provider
Study I (IHC)				
H_4R	Synthetic peptide, first cytoplasmic domain of human H_4R	Rabbit polyclonal	0,5 µg/ml	MBL Int.
Study II (IHC, IF)				
HDC	492-506 fragment of human HDC in C-domain of 53kDa isoform	Mouse monoclonal	1:350	F. Sanchez-Jimenez[1]
DAO	169-415 fragment of human DAO (HYB313-03)	Mouse monoclonal	1:500	H. Schwelberger[2]
HNMT	Synthetic peptide, N-terminal region of human HNMT	Rabbit polyclonal	2,5 µg/ml	Abcam
OCT2	Synthetic peptide, C-terminal sequence of human OCT2	Rabbit polyclonal	1:100	A. Nies[3]
OCT3	Synthetic peptide, C-terminal sequence of human OCT3	Rabbit polyclonal	1:100	A. Nies[3]
SMA	Synthetic peptide, N-terminal sequence of human SMA	Goat polyclonal	5 µg/ml	Everest biotech
Study III (WB)				
cPARP	p25 cleaved form of human PARP	Rabbit monoclonal	3,8 ng/ml	Abcam
ERK1/2	Synthetic peptide corresponding to 336-356 fragment of human ERK1	Mouse monoclonal	0,25 µg/ml	Millipore
pERK1/2	Phosphopeptide containing the pTEpY motif of human ERK1/2	Mouse monoclonal	0,1 µg/ml	Millipore
JNK	GST fusion protein corresponding to full-length rat SAPK1a	Rabbit polyclonal	1 µg/ml	Millipore
pJNK	Peptide corresponding to human pJNK at T183/Y185/T221/Y223	Rabbit polyclonal	1 µg/ml	Millipore
Bcl-XL	Synthetic peptide corresponding to 1-100 fragment of human Bcl-XL	Rabbit monoclonal	0,128 µg/ml	Abcam
BAX	Synthetic peptide corresponding to 1-100 fragment of human BAX	Rabbit monoclonal	0,114 µg/ml	Abcam
Unpublished data (IHC, IF)				
H_1R	Synthetic peptide, second extracellular domain of human H_1R	Rabbit polyclonal	0,5 µg/ml	MBL Int.
H_2R	Synthetic peptide, third extracellular domain of human H_2R	Rabbit polyclonal	0,5 µg/ml	MBL Int.
H_3R	Synthetic peptide, third cytoplasmic domain of human H_3R	Rabbit polyclonal	0,5 µg/ml	MBL Int.
H_4R	Synthetic peptide corresponding to a fragment of human H_4R	Rabbit polyclonal	0,497 µg/ml	P. Chazot[4]
moH_4R	Synthetic peptide corresponding to a fragment of mouse H_4R	Rabbit polyclonal	0,452 µg/ml	P. Chazot[4]

[1]Department of Molecular Biology and Biochemistry – Unit 741 of CIBERER, University of Málaga, Málaga, Spain.

[2]Department of Visceral, Transplant and Thoracic surgery, Molecular biology laboratory, Medical University of Innsbruck, Innsbruck, Austria (Schwelberger *et al.* 2013).

[3]Dr Margarete Fischer-Bosch institute of Clinical Pharmacology and University of Tübingen, Stuttgart, Germany

[4]Integrative Neuroscience, School of Biological and Biomedical Sciences, Durham University, Durham, United Kingdom.

6.7. Immunohistochemical staining (I, II)

4-µm sections of labial salivary glands (LSG) were deparaffinised and rehydrated. Antigen retrieval was performed in MicroMED T/T Mega microwave processing labstation for histology (Milestone) at 95°C for 15 min in 10mM sodium citrate buffer, pH=6, followed by cooling to a room temperature for 30 minutes. Later, the slides were washed in tap water and incubated in peroxidase-blocking solution (Dako) for 15 minutes, followed by 5 minutes wash in PBS. Epitope blocking was done by incubation in 10% normal goat/horse serum (Vector) for 1 hour at room temperature. Then, listed above primary antibodies were applied to the sections overnight at +4°C. Non-immune 0,5-2,5 µg/ml rabbit or mouse IgG were used for negative control staining. Next morning, the slides were washed in PBS 3 x 5 minutes, then incubated in appropriate biotin-conjugated secondary antibodies against certain primary IgG (1:200 in 1,25% BSA-PBS; Vector) for 1 hour, then incubated in avidin-biotin-peroxidase complex (1:200 in water; Vector) for 45 minutes. Colour reaction was developed by 0,006% hydrogen peroxide and 0,023% 3,3´-diaminobenzidine tetrahydrochloride (DAB) or metal enhanced DAB for 5-7 minutes. The slides were counterstained in haematoxyline or fast-red, dehydrated, mounted and covered. The sections were photographed using a Leitz Diaplan microscope and a 5MP Leica DFC420 digital camera (Leica Microsystems). In study II, human kidney tissue sections were used as positive sample controls (DAO staining).

6.8. Histamine transport studies (II)

The study was performed by our co-workers in M. Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, DE and original protocol described in our co-worker's publication (Nies *et al.* 2011).

The studies were performed as follows: Briefly, all cultures were incubated at +37°C. HSG cells ($5x10^5$/well) were seeded into 24-well cell culture plates and grown for 24 hours. Next day, the cells were washed with uptake buffer (130 mM NaCl, 25 mM hydroxyethylpiperazine ethanesulfonic acid, 1.2 mM KH_2PO_4, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, pH 7.4). Uptake was initiated by replacing this with uptake buffer supplemented with 100 nM [^{3}H]histamine (dihydrochloride, 392 GBq/mM; PerkinElmer, Waltham, MA), without and with 2 mM 1-methyl-4-phenylpyridinium (MPP), a model cation which inhibits organic cation transporters. Three washes in ice-cold uptake buffer stopped the uptake at

indicated time points, followed by lyses in 0.2% SDS. Intracellular radioactivity was determined by liquid scintillation counting (Hidex 300SL TDCR liquid scintillation counter, Turku, Finland). In some experiments, NaCl in the uptake buffer was replaced with 260 mM glucose.

For efflux studies, cells were pre-incubated for 60 min in uptake buffer containing 100 nM [^{3}H]histamine and then washed 3 times with ice-cold uptake buffer. Efflux was initiated by addition of uptake buffer (without [^{3}H]histamine). Radioactivity ([^{3}H]histamine) in the cell culture medium and cells was measured as above at the time points of 0.5 – 1 – 2 – 5 – 10 – 20 and 30 min.

6.9. Immunofluorescence staining (II, IV)

Snap-frozen 4 μm thick sections of LSG and murine salivary glands were incubated in 10% normal donkey serum for 1h at room temperature. Then, for histamine transport studies generously provided by our co-workers OCT2 (Nies *et al.* 2008) and OCT3 (Nies *et al.* 2009) antisera combined with SMA antibodies, for murine and additional H_4R studies generously provided by our co-workers in-house raised anti-mouse H_4R antibodies (Lethbridge and Chazot 2010) and in-house raised anti-human H_4R (van Rijn *et al.* 2006) were applied to the sections overnight at +4°C, followed by next morning 3 x 5 min PBS wash. After that, appropriate mixture of secondary antibodies (Do anti-Rb AlexaFluor 488-conjugated, Do anti-Go AlexaFluor 568-conjugated, Go anti-Rb AlexaFluor 568-conjugated, dilution 1:200 in PBS) were applied to the sections for 1 hour at room temperature, followed by another 3 x 5 min PBS-wash. Nuclear counterstaining with DAPI was done for 5 min at room temperature, followed by 3 x 5 min wash in distilled water. The sections were mounted and photographed by Leica DM6000 microscope and DFC365FX digital camera (Leica Microsystems, DE). Immunogen-containing absorption controls confirmed specificity of OCT2 and OCT3 antisera.

6.10. Immunofluorescence visualization of H_4R internalization in NS-SV-AC cells (III)

The cells were seeded on glass coverslips into plastic 24-well plate ($1x10^5$ cells/well) and cultured as previously described in section 6.4. Later, the cells were once gently washed with pre-heated 37°C PBS, followed by gentle change to 100 nM HST-10-containg K-SFM without BPE. Parallel cell sets were pre-treated with 10

mg/ml MβCD (Sigma, DE) for 1 hour prior to HST-10 stimulation. The cells we fixed in -20°C absolute methanol for 10 minutes after 5, 30, 60 and 180 minutes HST-10 stimulation, followed by 3 x 5 min wash in PBS. Fixed cells were incubated in 10% donkey normal serum for 1 hour at room temperature. After that H4R antibodies were applied to the cells for overnight at +4°C, followed by 3 x 5 min wash in PBS. Secondary Do anti-Rb AlexaFluor 488-conjugated antibodies were applied for 1 hour at room temperature, also followed by 3 x 5 min PBS wash. Nuclear counterstaining with DAPI was done for 5 min at room temperature, followed by 3 x 5 min wash in distilled water. The coverslips were mounted, visualized by Leica DM6000 microscope and photographed by DFC365FX digital camera. Negative control (0,5 µg/ml non-immune Rb IgG, R&D systems, MN) confirmed specificity of the primary antibodies.

6.11. Apoptosis induction (III)

Initial seeding density of NS-SV-AC cells was $3{,}5x10^5$ per well in 6-well plates. The cells were stimulated 8 hours prior induction of apoptosis with 5, 25, 50 and 100 nM HST-10 diluted in K-SFM, which didn't contain BPE. Apoptosis was induced by 100 ng/ml rhTNFα and 125 nM NF-κB inhibitor IMD-0354. The last one was required to sensitize the cells to TNFα-induced cell death. 100 nM staurosporine (Merk, DE) was used to induce positive control apoptosis for Western Blot experiments.

6.12. Phase contrast microscopy and Flow cytometry (III)

The apoptosis of NS-SV-AC cells at the time point of 36 hours was observed and analysed using Leica DM6000 microscope and photographed by DEC420 colour camera.

After that, the cells were collected and labelled with Alexa Fluor 488 Annexin V and propidium iodide from Dead Cell apoptosis kit for flow cytometry, according to manufacturer's instructions (Invitrogen, UK). Fluorescence emission at 530 nm and 575 nm was measured by BD Accuri C6 flow cytometer (BD Bioscience). Each sample was analysed for 20.000 events by the software provided to the cytometer.

6.13. Western Blotting (III)

Apoptotic and control NS-SV-AC cells were collected at the time points of 2,5-5-15-30-60 and 150 minutes for analysis of MAPKs and at the time point of 36 hours for analysis of cPARP, Bcl-XL and BAX. After pelleted by centrifugation at 1500 g cells were lysed by cell lysis buffer (Cell Signalling, MA) for 15 minutes on ice with agitation, then briefly sonicated and centrifuged at 14.000 g, +4°C for 10 min. Supernatant was collected and total protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific, IL). The lysis buffer contained 0,1% SDS, protease inhibitors (Roche, DE) and phosphatase inhibitors (Thermo Scientific, IL).

SDS polyacrylamide gel electrophoresis was performed according to a modification of the method by Laemmli, using 1,5 mm TGX Stain-Free Fast Cast 10% polyacrylamide slab gel (Bio-Rad, CA). Total protein amount of the samples was adjusted to 9-22 µg/lane, depending on efficiency of binding affinity of the primary antibodies. Running sample mixture was comprised of sample in 6X Red Loading Buffer and 30X DDT (New England BioLabs Inc, MA). The proteins in the sample mixture were denatured at +95°C for 10 minutes and plied to the gels, followed by electrophoresis and blotting to PVDF membranes. Then the membranes were washed in TBS for 10 minutes. Non-specific epitopes were blocked by 3% BSA blocking buffer for 1 hour at room temperature. Membranes were incubated in the above-listed primary antibodies at +4°C overnight either in PBS ± 0.1% Tween-20 or TTBS ± 5% milk / 2% BSA. Next morning the membranes were washed 3x10 minutes in TTBS and appropriate goat anti-rabbit/mouse HRP-conjugated secondary antibodies were applied for 1 hour at room temperature. The membranes were again washed 4x10 minutes in TTBS and HPR binding sites were detected by chemiluminescent reaction using Bio-rad Immun-Star WesternC Kit and Bio-rad ChemiDoc MP Imaging system. Intensity of the bands was normalized to stain-free total protein loading controls and analysed by Bio-rad Image Lab software.

6.14. Quantitative Real-time PCR (I, II, III, IV)

Salivary gland total RNA was isolated from frozen samples using a High Pure RNA Tissue kit (Roche, CH). Apoptotic and control NS-SV-AC cells were pelleted at 14.000 g at +4°C for 10 min at the time point of 20 hours after initiation of apoptosis. Total RNA from NS-SV-AC cells was isolated using RNeasy Plus Mini kit (Qiagen,

DE). Complementary DNA (cDNA) was synthesized using SuperScript First Strand cDNA synthesis kit (Invitrogen, CA).

Total HSG cell RNA was extracted using the MirVana miRNA isolation kit (Ambion, US). cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (LifeTech, MA).

For human H_4R, HDC, DAO, HNMT, housekeeping human β-actin, housekeeping human GAPDH and housekeeping murine β-actin gene transcripts, quantitative RT-PCR was performed using the iCycler iQ5 Multicolor Real-Time PCR Detection System and iQ SYBR Green Supermix Kit (Bio-rad). Sequences of primers are listed in the table below. Expression levels of human OCT1, OCT2, OCT3 and PMAT mRNAs were quantified by TaqMan technology on 7900HT RT-PCR system (LifeTech). Expression levels of murine H_4R mRNA were quantified by TaqMan technology on iCycler iQ5 Multicolor Real-Time PCR Detection System and FAM-labelled iTaq DNA polymerase kit (Bio-rad).

Primers used in qRT-PCR experiments

Name	Sequences of the primers	Study
H_4R	Forward: 5'-TGG AAG CGT GAT CAT CTC AG-3' Reverse: 5'-ATA TGG AGC CCA GCA AAC AG-3'	I
HDC	Forward: 5'-TTG ATT GCC CTG CTG GCA GC-3' Reverse: 5'-TGC ACA GAC AAA GAC GGG CAC C-3'	II
HNMT	Forward: 5'-TGG CAT CTT CCA TGA GGA GCT T-3' Reverse: 5'-AAA ATC CCA AAG CAG GTC TCC AT-3'	II
DAO	Forward: 5'-GCT ACG TCC ACG CCA CCT TCT A-3' Reverse: 5'-CCC AGG CCA CCA GGT CCT CA-3'	II
Bcl-xL	Forward: 5'-GAT CCC CAT GGC AGC AGT AAA GCA AG-3' Reverse: 5'-CCC CAT CCC GGA AGA GTT CAT TCA CT-3'	III
BAX	Forward: 5'-CGG GTT GTC GCC CTT TTC TA-3' Reverse: 5'-GTC CAA TGT CCA GCC CAT GA-3'	III
β-actin	Forward: 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3' Reverse: 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3'	I, II
GAPDH	Forward: 5'-AAG GTC ATC CCT GAG CTG AA-3' Reverse: 5'-TGC TGT AGC CAA ATT CGT TG-3'	III
OCT1	Forward: 5'-CAT CAT AAT CAT GTG TGT TGG CC -3' Reverse: 5'-CCC TTG GTC TCT GGA AGA AGT AG-3'	II
mouse β-actin	Forward: 5'-CTT CTT TGC AGC TCC TTC GT -3' Reverse: 5'-GTG CCA GAT CTT CTC CAT GT -3'	IV

6.15. Statistical analysis (I, II, III, IV)

Quantified data are presented as mean ± SEM. Online two-tailed Mann-Whitney *U* test was used for comparisons and significance (skewed qRT-PCR data in the studies I, II, IV). Online one-way ANOVA or MS Excel Student's *t*-test were used to estimate statistical significance of differences between groups with normally distributed data (ELISA results in the study I; Flow cytometry data, Western Blot data and qRT-PCR data in the study III). A p-value of 0.05 or less was considered statistically significant.

7. RESULTS AND DISCUSSION

7.1. Histamine receptors profile in the salivary glands

At the very beginning of the project, we hypothesized, that H_4R might be expressed in the salivary glands of healthy human individuals and somehow contributes to pathogenesis of SS. Additionally, in order to determine possible histamine-mediated effects in the human salivary glands in SS, the convectional H_1R, H_2R as well as H_3R were also studied. Immunohistochemistry clearly revealed the H-receptor expression profiles of the human salivary glands (Figure 7).

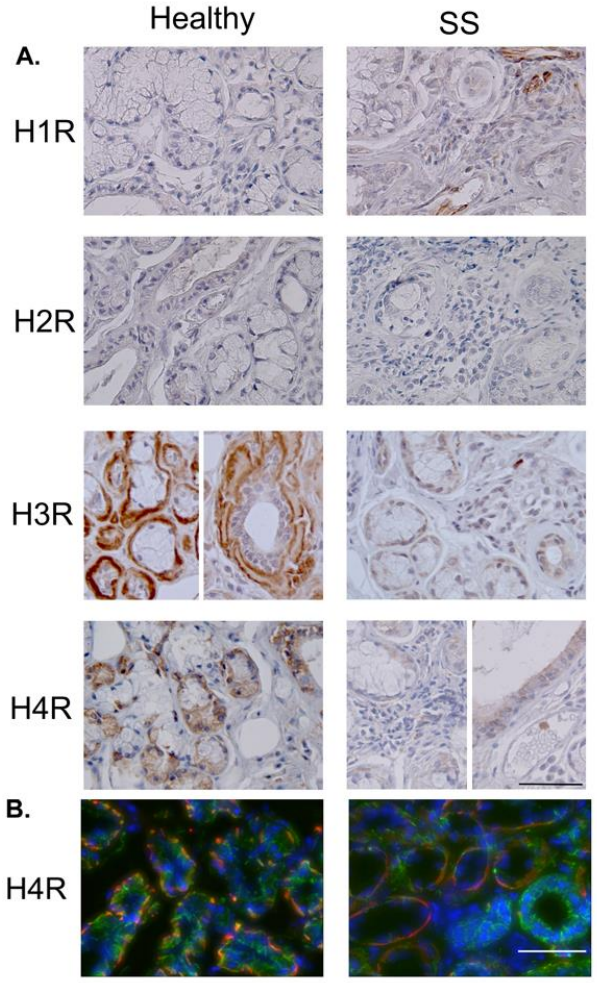


Figure 7.
A. IHC staining of H-receptors in the salivary glands from healthy individuals and SS patients.
In healthy salivary glands H_1R was localized only in the endothelial cells of the blood vessels. H_2R was not detected by IHC. H_3R was localized in the myoepithelial cells around the acini and ducts. H_4R localized in the acinar and in ductal epithelial cells. IHC revealed no difference in expression of H_1R and H_2R in SS samples as compared to healthy samples. Both H_3R and H_4R were stained weaker in SS samples as compared to samples of healthy salivary glands.

B. IF staining of H_4R using antibodies provided by prof. P. Chazot. Membrane staining of H_4R can be observed in healthy acini, whereas acini in SS are H_4R-negative.
Bars = 50 μm

According to the results of immunohistochemical staining, localization of H_1R in both healthy and SS biopsies was restricted to blood vessel endothelium and solitary lymphocytes of inflammatory foci in SS samples. This finding is in line with studies of others and dogma of H_1R-mediated vascular dilatation and permeability (Van de Voorde and Leusen 1983; Li *et al.* 2003). However, the observed results were contradictory to those of Kim J-H. *et al*., who showed H_1R expression "distributed across most of the human submandibular gland including acinar and ducts" (Kim *et al.* 2009), which might result from cross-reactivity or false-positive staining. H_2R protein was not immunohistochemically detected neither in healthy salivary biopsies, nor in biopsies from SS patients.

The most intriguing results of IHC staining were observed regarding H_3R and H_4R. In healthy salivary glands localization of H_3R corresponded to myoepithelial cells around the acini and the ducts. H_4R was localized in the acinar cells and in the goblet cells of the ducts. Immunoreactivity of both H_3R and H_4R was lower in most of SS samples as compared to healthy samples. Results of qRT-PCR seemed in line with those of IHC, since expression of H_4R were relatively low in SS samples compared to healthy samples at mRNA level, but the difference did not reach statistical significance. Positive myoepithelial staining pattern of H_3R may be a result of expression of the receptor on the surface of myoepithelial cells and/or due to expression on the surface of cholinergic fibres, which innervate myoepithelial cells. This finding is in accordance with those of investigators, who showed that regardless of the predominant localization in the central nervous system, H_3R is also expressed in the peripheral nerve fibres inhibiting local cholinergic transmission (Ichinose and Barnes 1989; Cannon *et al.* 2007). H_3R localization is also demonstrated in a number of muscular cells, such as bronchial smooth muscle cells (Cardell and Edvinsson 1994), bladder detrusor cells (Neuhaus *et al.* 2006) and even in the skeletal myotubes (Chen *et al.* 2015). The impact of H_3R in salivary gland maintenance and pathogenesis of SS is still an open question and interesting approach for future studies.

H_4R immunohistochemical reactivity was localized in the acini and in the goblet cells of the ductal epithelium in healthy LSG. H_4R immunoreactivity in most of SS LSG samples was visibly lower, compared to healthy samples (Figure 7, A). Use of second in-house raised antibodies against human H_4R (a generous gift from prof. P. Chazot) revealed clear membrane localization of H_4R in IF staining (Figure 7, B).

These findings encouraged us to continue studies towards finding the role of histamine/H_4R in SS.

Murine NOD lines are often used as animal models for SS studies. Salivary glands of NOD mice are characterized by similar lymphocytic infiltrates to those observed in SS (Braley-Mullen and Yu 2015). Parallel immunofluorescence studies revealed clear presence of moH_4R in the murine salivary glands. Interestingly, moH4R was present exclusively on the surface of the apical membrane of acinar and ductal epithelia as compared to human samples, where an all-over membrane localization of the receptor was observed (Figure 8). Unfortunately, there was no significant difference of the moH_4R expression pattern in the salivary glands of the SS model NOD line compared to the healthy control BALB line. Thus, most of further studies were performed using biological materials from human species.

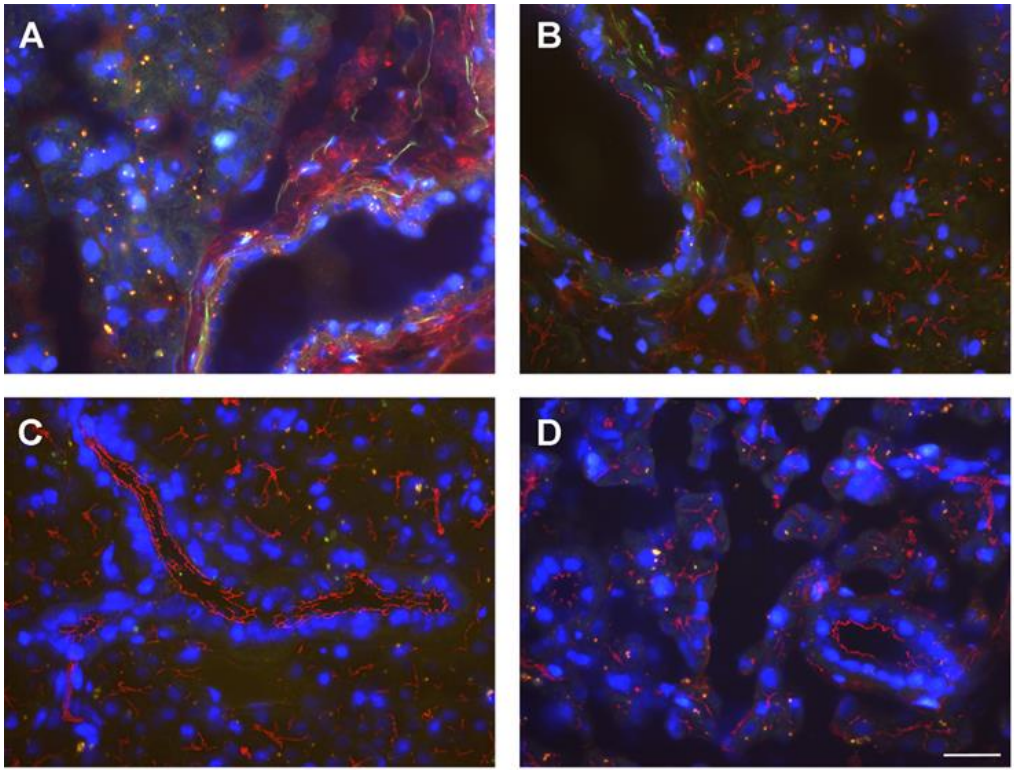


Figure 8. Immunofluorescence staining of moH4R in murine salivary glands. The receptor (red colour) was localized with sharp apical staining pattern in the acinar (star-like patterns) and ductal epithelial cells of the gland. A = Negative control (1-month-old BALB line). B = control 1-month-old BALB line. C = 4-months-old NOD line. D = 7-month-old NOD line. Bar ≈50 μm

7.2. Histamine transport and metabolism in the salivary glands (II)

7.2.1. Histamine metabolizing enzymes in LSG

Exclusive histamine synthesizing enzyme HDC and intracellular histamine degrading enzyme HNMT were found in the healthy LSG. Immunoreactivity of those enzymes was found in the ductal epithelium, predominantly in the intralobular excretory salivary ducts of both healthy and SS LSG without significant difference in protein expression pattern. Extracellular histamine degrading enzyme DAO was not found in either of groups of samples. These results were completely supported by qRT-PCR.

Based on the mentioned above results of IHC and qRT-PCR, it is reasonable to propose, that local synthesis and degradation of histamine in the salivary glands are not altered in SS patients.

7.2.2. Histamine transporters in LSG

mRNA levels of major histamine transporters OCTs and PMAT were measured in the salivary glands of healthy- and SS individuals. OCT3 mRNA was present at levels over 100-fold higher than the rest of tested transporters. Healthy LSG samples showed significantly higher expression of OCT3 as compared to SS samples ($p=0.028$). This result supports OCT3 as a major histamine transporter in salivary glands. Later, IF staining showed strong basolateral membrane localization of OCT3 in the epithelial cells of healthy LSG (Figure 9). SS samples were almost OCT3-negative in IF staining, supporting results of qRT-PCR. These results suggest, that transport of histamine via OCT3 is altered in the acinar and ductal epithelia in SS.

Double IF staining revealed co-localization of OCT2 and smooth muscle α-actin in the myoepithelial cells of salivary glands from healthy individuals. SS samples showed much weaker expression of OCT2, as compared to healthy samples.

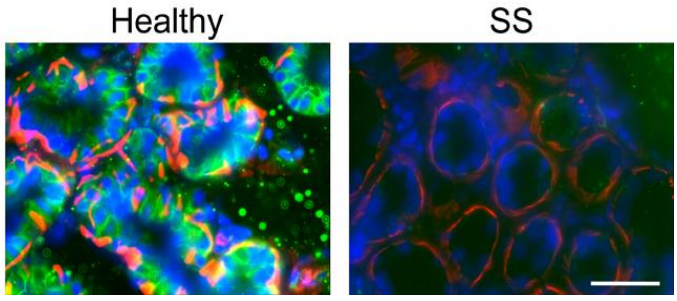


Figure 9.
Representative IF figures of OCT3 in the salivary glands 6 healthy controls and 8 SS patients with similar results.
Bar = 50

7.2.3. Histamine transporters in HSG cells

In qRT-PCR experiments, HSG cells expressed OCT3 mRNA at a level of over 10-fold higher than OCT1 and OCT2, and over 2-fold higher than ENT4/PMAT, supporting a leading role for OCT3 in histamine transport in HSG cells. Preloaded HSG cells were able to release [^{3}H]histamine into histamine-free medium in a time-dependent manner. Conversely, HSG cells showed also time-dependent [^{3}H]histamine uptake from the loading buffer, but not in the presence of OCT3 inhibitor MPP.

7.2.4. OCT3 in murine salivary glands

Immunofluorescence staining revealed OCT3 in the murine salivary glands. Three types of glands were stained: submandibular, parotid and sublingual. OCT3 was observed in the acinar and ductal epithelia in submaxillary and sublingual salivary glands. The staining signal was restricted to the apical membranes of the cells of the salivon, appearing as a star-like staining pattern. Additionally, OCT3 strong immunoreactivity was observed in the round-shaped structures, which could be identified as blood vessels and also in the solitary spindle-like cells in stroma of the glands, which could be mast cells and/or dendritic cells. Immunoreactivity of OCT3 in the parotid gland was mostly observed in the apical membrane of ductal cells, whereas acinar staining was weak, as compared to sublingual and submandibular glands (Figure 10).

Submaxillary gland in mice is homologous to human submandibular gland, and is considered a mixed gland, because it contains both serous and mucous acini. Together, mainly mucus sublingual gland and submaxillary gland are major sources of the watery compound of total saliva. Thus, staining data suggest that in the salivary

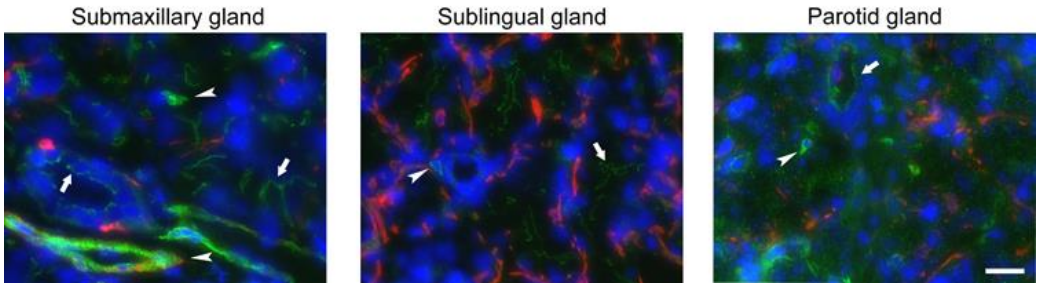


Figure 10. Double immunofluorescence staining of different salivary glands of 7-month-old NOD mouse. Green color corresponds to OCT3, red color corresponds to smooth muscle α-actin, blue color corresponds to nuclei (DAPI). OCT3 was expressed in the apical membrane of the acinar (star-like staining pattern) and ductal epithelia (arrows), as well as in the blood vessels and the cells of stroma, presumably in the mast cells and/or dendritic cells (arrowheads). Bar ≈ 50 μm.

glands of NOD mice OCT3 is restricted to mucous-synthesizing epithelial cells, rather than to protein-rich serous protein-synthesizing cells. Apical membrane star-like staining pattern of OCT3 refers to co-localization with H_4R in mice as well. Studies show, that apical membrane of the murine mucous salivary epithelium is equipped with water channel protein aquaporin-5 (AQP-5). The saliva secretion in AQP-5 knock-out mice decreases significantly (Ma *et al.* 1999). Taking into account restricted apical membrane co-localization of H_4R/OCT3/AQP-5, it can be speculated that H_4R/OCT3-mediated effects of low-concentration histamine may play role in secretion of water into saliva via AQP-5 in mice.

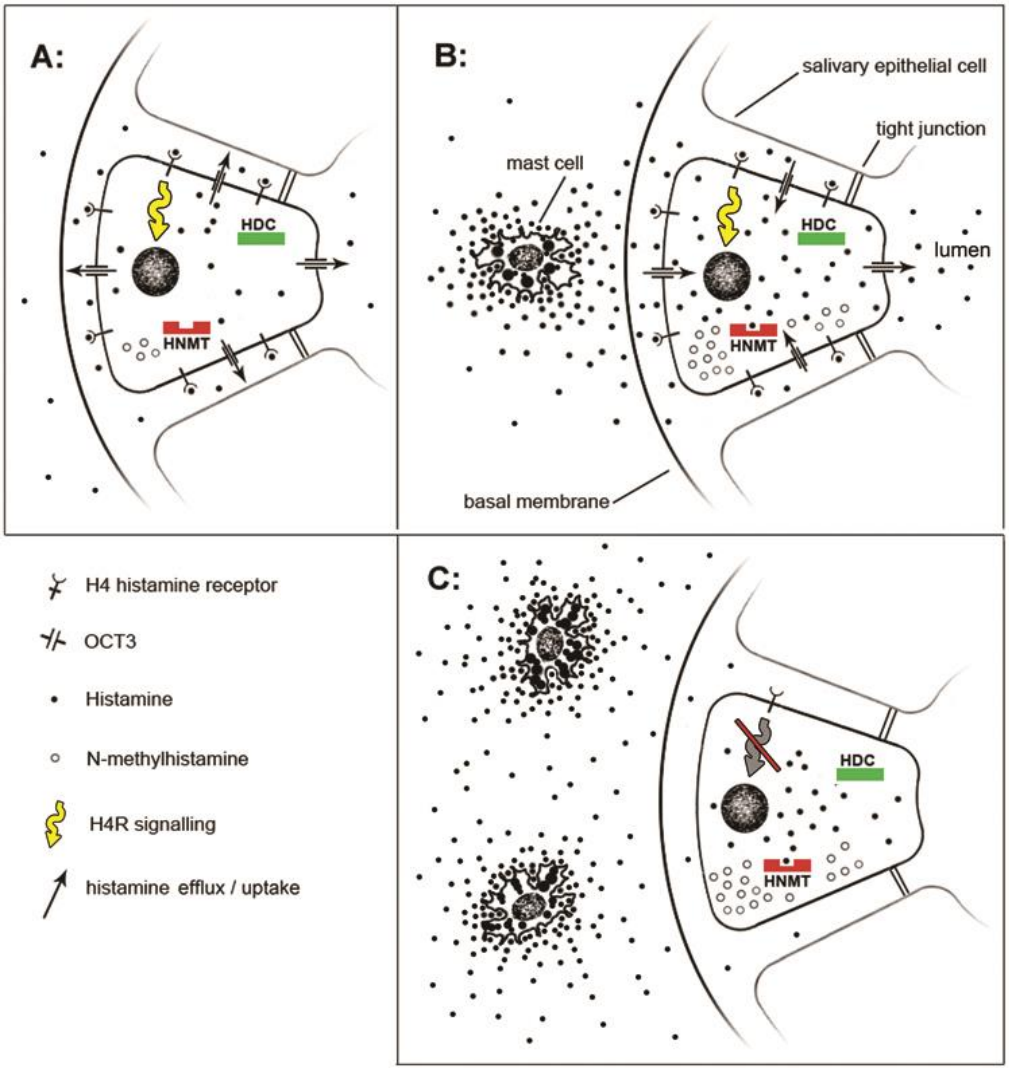


Figure 11. Different conditions of the salivary gland epithelium
A: Healthy salivary gland at resting "off-state". Salivary epithelium continuously synthesizes and releases histamine via OCT3. H_4R is activated.
B: Healthy salivary gland at active "on-state". Epithelial cells uptake excessive amounts of histamine from the mast cells for degradation and secretion into the lumen.
C: Salivary gland in Sjögren's syndrome. Salivary epithelium is H_4R and OCT3-negative. Mast cells release histamine, which is not uptaken by epithelial cells and promote H_1R-mediated pro-inflammatory effects.
HDC = histidine decarboxylase, HNMT = histamine N-methyltransferase

7.3. Non-professional histamine producing cells and on/off-state conception in SS

Professional histamine producing cells, such as mast cells, basophils and ECL-cells of the gastric mucosa utilize 53-kDa HDC with high enzymatic activity to synthesize histamine rapidly into the storage granules, from which it is released in a burst-like manner in order to reach momentary high local histamine micromolar concentrations (Ichikawa *et al.* 2010). These concentrations are enough to activate conventional H_1R and H_2R. Vast quantities of rapidly released histamine and subsequent H_1R/H_2R-mediated effects are responses to alteration of a tissue, as they take part in various pathophysiological processes like allergy, acute inflammation, infection etc. This "on-state" response is rapid, short-term and required to eliminate etiological factors. In turn, non-professional histamine producing cells, such as epithelial cells, utilize immature 74-kDa isoform of HDC with low enzymatic activity, continuously synthesizing and passively releasing histamine in low nanomolar concentrations into the extracellular space. These concentrations are enough to activate high affinity H_3R and H_4R. We believe that "off-state" long-term H_3R/H_4R-meditated effects are required to maintain local homeostasis and physiological responses. Based on our results we suggest, that in healthy salivary gland tissues histamine-mediated effects of professional and non-professional histamine producing cells are in balance in a way that epithelial cells serve as "histamine sinks", which harbour unnecessary excessive histamine via OCT3, degrade it by HNMT and/or lead it out into the salivary lumen. Our studies showed that salivary glands are practically DAO-free, that makes extracellular histamine degradation rather unlikely. Thus, the epithelial OCT3/HNMT-mediated intracellular mechanism brings back homeostatic histamine quantity to the milieu (Figure 11, B). The studies show that number of histamine-containing mast cells positively correlates with the degree of local immune inflammation as assessed by focus score counting in SS (Konttinen *et al.* 1990). Apparently, local inflammatory surroundings drive mast cells to release histamine into the milieu, therefore contributing to pathogenesis of the disease as is shown, for example, in oral lichen planus (Salem *et al.* 2015). Salivary glands of SS patients are H_1R-positive. Activation of H_1R increases cytosolic calcium, which may result in activation of endothelial eNOS and production of NO. This leads to vasodilatation and increased vasopermeability, helping to recruit more immunocompetent cells from the blood stream into lymphatic foci in the diseased salivary gland. A new suggested

role for the OCT3-positive salivary epithelial cells in the intracellular degradation of histamine is that they are down-regulated in SS. This can contribute to prolonged "on-state" H_1R-mediated pro-inflammatory effects (Figure 11, C). *In vitro* histamine transport studies in HSG cells supported this conclusion. Together with immunostaining results and qRT-PCR results, inhibition of [^{3}H]histamine uptake in presence of MPP suggests a meaningful role of OCT3 histamine uptake in human salivary glands.

It was very exiting to discover co-localization of OCT3/H_4R and OCT2/H_3R as a cell-type-specific compartmentalization within one functional unit of the gland. Especially, when OCT3/H_4R co-localization was also observed in salivary glands from NOD mice. It may be assumed that histamine participates/regulates contractile function of the myoepithelial cells via co-localized OCT2 and H_3R. At the same time, acinar, ductal and myoepithelial cells can uptake histamine via OCT2/3 for intracellular degradation, intracellular sequestration and/or salivary secretion. Histamine concentration in saliva is 0.31-12,4 ng/ml (2.8-112.7 nM) (Kejr *et al.* 2010), which is exactly enough to activate high affinity H_3R and H_4R (K_i H_3R = 15,8 nM, K_i H_4R = 7,9 nM) (Thurmond *et al.* 2008). In SS the basal maintenance of the resting "off-state" status seems to be disturbed due to reduced OCT3 and H_4R expression in the acinar and ductal epithelial cells. Thus, observed deficiency of OCT2 and H_3R can also contribute to SS pathogenesis as well, for instance, by impaired regulation of salivary flow.

Many cells of the lymphocyte infiltrates in SS are H_4R-positive non-professional histamine-producing cells. Among those are antigen-presenting dendritic cells, T- and B-lymphocytes (Zampeli and Tiligada 2009). Therefore, deranged histamine transport and degradation in salivary glands may also have effects on local immune reactions and lymphocyte infiltration in SS.

7.4. H_4R in the salivary gland cell cultures

7.4.1. H_4R regulates production of IL-8 and VEGF in HSG cells

xMAP Multiplex screening with subsequent ELISA validation showed, that HSG cells are able to secrete IL-8 and VEGF. Accumulation of both cytokines was time-dependent. 10 μM ST-1006-stimulated HSG cells secreted IL-8 and VEGF at higher concentrations compared to non-stimulated controls (Table 4).

Table 4. Accumulation of IL-8 and VEGF in the supernatant of ST-1006-stimulated HSG cells *vs.* non-stimulated controls. Number of independent experiments = 3.

Time points, hours	**IL-8**, ng/ml		**VEGF**, ng/ml	
	non-stimulated control supernatant	ST-1006-stimulated supernatant	non-stimulated control supernatant	ST-1006-stimulated supernatant
4	0,7±0,3	0,6±0,2	1,9±0,2	1,6±0,4
8	0,8±0,4	1,5±0,3	1,9±0,3	2,1±0,3
12	0,9±0,3	1,8±0,2*	2,2±0,2	2,6±0,5
16	1,0±0,2	1,5±0,4	2,8±0,4	3,3±0,2
24	1,2±0,3	1,6±0,4	3,3±0,3	4,5±0,3*
32	1,4±0,2	2,0±0,3*	3,8±0,4	4,8±0,2*
48	2,0±0,4	3,2±0,5*	4,2±0,4	7,0±0,4*

* = $p<0.05$ in comparison to non-stimulated control.

In this study we used 10 μM H_4R specific agonist ST-1006 (E_{max} = 28,4 %) to stimulate HSG cells. Such concentration is about 1000-fold higher than dissociation constant of ST-1006 (K_i $h$$H_4R$ = 12 ± 3 nM) (Sander *et al.* 2009), what mimics micromolar histamine concentrations released from the activated mast cells into the milieu in the salivary glands of SS patients. Similar to SS, mast cell accumulation and degranulation was also demonstrated by Salem and co-workers in oral lichen planus, an autoimmune disorder characterized by oral mucosa lesions. It was also shown, that H_4R was significantly down-regulated at mRNA level after 24 h exposure to 50 μM histamine and 10 ng/ml IFN-γ in squamous cell carcinoma cell line (Salem *et al.* 2015). Based on this it can be speculated, that in SS patients H_4R might also be down-regulated influenced by an inflammatory milieu and by an excessively saturated mast cell-derived histamine load. As a result, H_4R-mediated local homeostatic maintenance of the desensitized epithelial cells is imbalanced. As a consequence, this may trigger an increased production of harmful cytokines.

Results of this study support those of Lee *et al.*, who demonstrated by use of Multiplex assay, increased levels of IL-8 in saliva in patients with primary SS (Lee *et al.* 2010) as well as in serum of patients with secondary SS (Eriksson *et al.* 2004; Hwang *et al.* 2014), but not in plasma (Szodoray *et al.* 2004). IL-8 (CXCL8) is a

chemotactic factor, which attracts neutrophils and other granulocytes towards the site of infection (Rossi and Zlotnik 2000).

IL-8 is also known as a promoter of angiogenesis and together with VEGF theoretically can cause neovascularization of the salivary glands. Increased blood flow in the salivary glands of some SS patients has been reported (Steiner *et al.* 1994; Chikui *et al.* 2000) and more recently pro-angiogenic factors were studied (Szodoray *et al.* 2004; Delaleu *et al.* 2008; Sisto *et al.* 2012). However, the analysis of vascular organization of SS salivary glands for signs of angiogenesis resulted in controversial results between two research groups. By IHC sole staining of platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), hematopoietic progenitor cell antigen CD34, macrophages and histiocytes Sisto *et al*. showed increased neovascularization in association with the grade of inflammatory lesions in SS minor salivary glands (Sisto *et al.* 2014). In contrast, McCall *et al*. in immunofluorescence double staining of PECAM-1 and von Willebrand Factor demonstrated unchanged organization of blood vessels in SS, supported by unchanged levels of VEGF in SS salivary glands vs. controls in western blot experiments (McCall and Baker 2015). Both groups used the same standard histological focus score grading analysis of the specimens. Therefore, there is no solid agreement today on the role of VEGF in SS and this issue is still a matter of studies and debates.

7.4.2. Internalization of H_4R in NS-SV-AC cells

NS-SV-AC cell line derives from isolated human salivary gland acinar epithelial cells (Azuma *et al.* 1993). This cell line exhibits acinar phenotype, which makes it more advantageous, compared to ductal phenotype HSG cells, which turn into acinar ones only upon culturing on matrigel. The purpose of this study was to check whether NS-SV-AC line express functional H_4R. At the same time another more effective specific H_4R agonist HST-10 (E_{max} = 85 %) became available to our disposal, and it was therefore used in the stimulation experiments. Internalization was detected by immunofluorescence staining and followed at several time points. Non-stimulated cells were weakly stained for H_4R. 100 nM HST-10-stimulated cells exhibited major staining patterns of the receptor's internalization phases: membrane (5 min), cytoplasmic/perinuclear (30 min), sequestration/disappearance of the signal (60 min), and recycling/reappearance of the signal (180 min). MβCD-pretreated cells exhibited delay in the internalization phases, showing that cytoplasmic staining still prevailed at

60 min and sequestration at 180 min (III, Fig. 1). Thus, we demonstrated that NS-SV-AC cells express H_4R, which was confirmed to be functional (as will be presented in paragraph 7.4.4 below), and MβCD inhibited clathrin-dependent internalization of H_4R. Weak staining of non-stimulated cells and disappearance of receptor labelling at the sequestration phase was probably due to ability of the antibodies to recognize 3D-conformational structure of the epitope in the 1st cytoplasmic domain of the receptor only in its ligand-coupled active state.

7.4.3. NS-SV-AC cells undergo TNFα/IMD-0354-induced apoptosis

To study if immortalized NS-SV-AC cells can undergo apoptosis, a combination of human recombinant TNFα with NF-κB inhibitor IMD-0354 as an apoptotic trigger was tested. The sole use of TNFα did not cause strong enough apoptosis even at concentration of 1 μg/ml. On the other hand, high micromolar concentrations of IMD-0354 were able to induce rapid apoptosis, but not via the extrinsic pathway, which is considered important in terms of SS pathogenesis. Pilot experiments determined the best synergistically effective apoptosis-inducing doses as being 100 ng/ml TNFα and 125 nM IMD0354, which were significantly ineffective in the sole experiments.

Phase contrast microscopy showed typical apoptotic morphology of TNFα/IMD-0354-stimulated NS-SV-AC cells, characterized by retraction of the cells, formation of apoptotic bodies and detachment from the surface. Control non-apoptotic healthy cells exhibited spindle-like shape without signs of structural changes/alterations in the cell membrane (Figure 12).

Flow-cytometry experiments showed increasedAnnexin V and PI double labeling of TNFα/IMD-0354-stimulated NS-SV-AC cells, taken as signs of apoptosis and

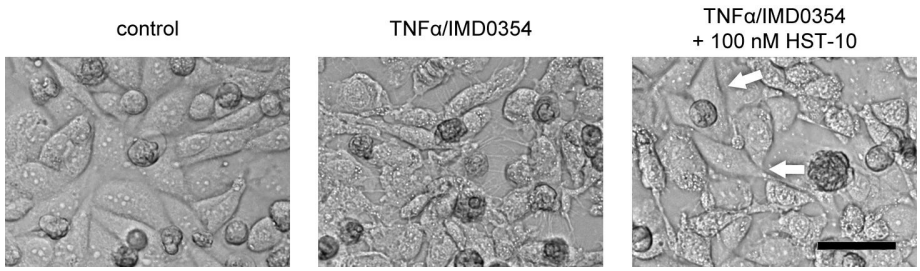


Figure 12. Phase contrast microscopy. Arrows point on live spindle-shaped NS-SV-AC cells in the presents of apoptosis-inducing TNFα/IMD-0354 mixture and 100 nM H_4R agonist HST-10. Bar = 50μm.

secondary necrosis. Prevalence of apoptotic cells in the experimental medium was also observed as changes in distribution of the cell population on the scatter SSC/FSC plot (Figure 13). Finally, TNFα/IMD-0354-induced apoptosis was confirmed by increased late phase apoptosis marker cPARP in Western blot experiments, as a result of caspase proteolytic cascade. Use of staurosporine as positive control confirmed apoptosis in immunoblots (III, Fig. 2c). Thus, suitable TNFα-mediated apoptotic model for NS-SV-AC cells was developed and used in subsequent experiments.

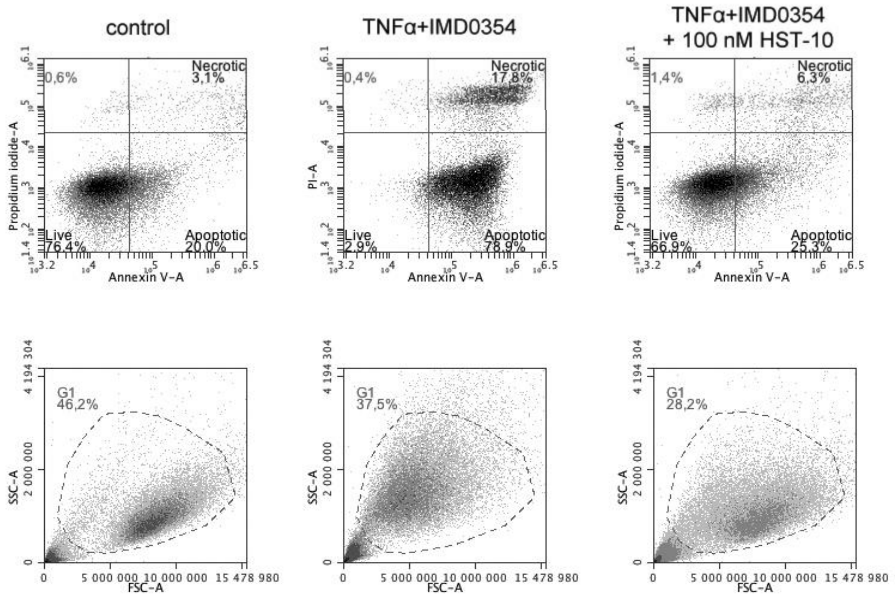


Figure 13. Fist row: apoptotic cells detected by flow cytometry using Annexin V and propidium iodide double staining. Second row: gated cell population in forward scatter (FSC) vs. side scatter (SSC) plot.

7.4.4. Anti-apoptotic activity of H_4R in NS-SV-AC cells

Activation of H4R by specific agonist HST-10 resulted in inhibition of TNFα/IMD0354-induced apoptosis of NS-SV-AC cells. Flow cytometry results showed decreased Annexin V and PI labeling of the apoptotic cells in the presence of HST-10 in the cell culture medium in a dose-dependent manner (Table 5). Statistically significant anti-apoptotic activity of HST-10 was reached at concentration of 100 nM. This dose was defined as the best effective dose and was used in all subsequent experiments. Percentage of the live cells in 100 nM HST-10-containing experimental medium was significantly higher as compared to non-stimulated apoptotic control: 63±6% *vs.* 11±7% (p=0.003). On the other hand, the percentage of apoptotic (Annexin-V-labeled) cells was significantly lower in 100 nM HST-10-containing experimental medium, as compared to non-stimulated apoptotic control cells: 25±6% *vs.* 66±13% (p=0.022), respectively (Table 5). Phase contrast microscopy visually disclosed increased numbers of preserved spindle-like shaped non-apoptotic NS-SV-AC cells in HST-10-containing medium after induction of apoptosis (Figure 12, arrows). Finally, the H_4R-mediated anti-apoptotic effect was confirmed in Western blot experiments that showed significantly decreased cleavage of PARP in HST-10-pretreated apoptotic cells (III, Fig. 2c).

Further experiments were performed to study which intracellular MAPK pathways might provide protection against TNFα/IMD0354-induced apoptosis upon H_4R activation. JNK and ERK MAPKs were analyzed using Western blot. TNFα/IMD0354-induced phosphorylation of both JNK and ERK in control NS-SV-AC cells was clearly observed at 2.5 min, followed by drop to initial levels after 30 min. HST-10 pretreatment caused inhibition of TNFα/IMD0354-induced JNK

Table 5. Percentage of the live and apoptotic cells in flow cytometry experiments. Values are presented in round numbers.

Cells	Control (n=4)	TNFα/IMD0354				
		apoptotic control (n=4)	5 nM HST-10 (n=3)	25 nM HST-10 (n=3)	50 nM HST-10 (n=3)	100 nM HST-10 (n=4)
Live, %	78±3	11±7*	26±11	36±12	48±10	63±6**
Apoptotic, %	17±3	66±13*	52±14	42±14	30±13	25±6**

p<0.05 compared to control, ** p<0.05 compared to TNFα/IMD0354 alone, n = number of independent experiments.

phosphorylation at 2.5 and 15 min, followed by rise to initial levels at 30 min (Figure 14). HST-10 treatment did not cause significant effect on ERK phosphorylation. Elevated levels of pJNK and pERK at 60 min and 150 min were interpreted as reflection of secondary changes in the MAPK signaling pathways, rather than H_4R-mediated.

Additionally, major members of Bcl-2 regulatory protein family were studied as

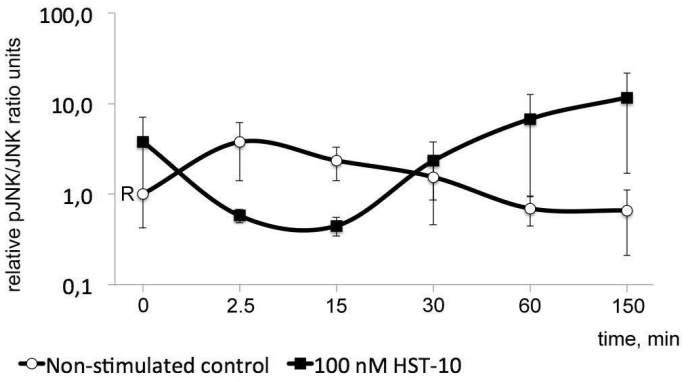


Figure 14.
Phosphorylation of JNK MAPK in TNFα/IMD0354-stimulated NS-SV-AC cells. Quantification of Western blots.
Presented as mean ± SEM of 3 independent experiments.
R = reference mean.

markers of mitochondrial link of apoptosis. Pro-apoptotic protein BAX and anti-apoptotic protein Bcl-XL were studied in TNFα/IMD0354-induced apoptosis in NS-SV-AC cells at mRNA and at protein levels. Influence of apoptotic TNFα/IMD0354 mixture upon the cells resulted in diminished levels of both BAX and Bcl-XL proteins at mRNA level after 20 h and at protein level after 36 h of treatment. H_4R activation with HST-10 resulted in statistically significant up-regulation of anti-apoptotic Bcl-XL at mRNA and protein level in TNFa/IMD0354-treated cell, while levels of BAX remained unchanged (III, Figure 4). Interestingly, active form ≈22 kDa BAX was not detected in Western blot experiments, probably due to Simian-virus-40-mediated suppression of BAX activation promoter p53 (Chipuk *et al.* 2004; Alwin Prem Anand *et al.* 2012; Lee *et al.* 2014). Instead, protein levels of BAX were analyzed based on inactive form of the protein (III, Figure 4e), which is suggested to be a dimer or small protein-coupled complex (Vogel *et al.* 2012). Presented data at least partly explains underlying molecular mechanisms of H_4R-mediated protective effects of histamine against TNFα-induced apoptosis in NS-SV-AC cells.

7.4.5. Role of H_4R in Sjögren's syndrome

Glandular epithelial cells are proposed to be a potent component in disease pathogenesis. Lymphocytic foci in the salivary glands in SS patients form and gradually enlarge around epithelial structures. The inherent abilities for intrinsic immunoreactivity of the salivary gland epithelium are supported by the presence of numerous molecules involved in antigen presentation (MHC I and MHC II), co-stimulation (CD40, CD80), cell adhesion (CD54, ICAM-1) and apoptosis (Fas, FasL) on the surface of the epithelial cells (Mavragani and Moutsopoulos 2014). Lymphocyte infiltrates in the diseased glands comprise autoreactive T-cells, which escape cell death due to a defective peripheral Fas/FasL-dependent mechanism of tolerance (Hayashi *et al.* 2004). This may cause T-cells polarization to IFNγ-producing T_h1 cells, being contributors to epithelial lesion in SS (Segerberg-Konttinen *et al.* 1987; Konttinen *et al.* 1999; Mitsias *et al.* 2002). Besides this, other immunocompetent cells, identified as T_h17, subset of NK-cells, also $CD8^+$ cells are found in the lymphocyte infiltrates. Such aggressive inflammatory milieu favors intensive apoptosis of the epithelial cells, turning them into a source of autoantigens. Epithelium-derived apoptotic bodies, microparticles and exosomes contain pathologically processed cryptic epitopes and immunogenic determinants. They are consumed by immature plasmacytoid dendritic cells and presented subsequently to T-cells. In turn, activated T-cells initiate B-cell polarization into autoantibody-producing plasma cells, therefore inducing/enhancing the vicious cycle of SS pathogenesis. Indeed, one of the diagnostic criteria for SS is the presence of serum autoantibodies directed against SS-A/B hY RNA ribonucleoproteins.

Taken together our results on altered H-receptor profile along with altered OCT3 transporter in the salivary epithelium in SS patients and recently reported anti-apoptotic features of submicromolar histamine in rodents (Medina *et al.* 2011; Carabajal *et al.* 2012; Prestifilippo *et al.* 2012), it was hypothesized that activation of H_4R may provide anti-apoptotic effect in salivary glands as well. Based on our results herein we report that activation of H_4R with nanomolar concentrations of selective receptor agonist HST-10 results in inhibition of TNFα/IMD0354-induced apoptosis in NS-SV-AC cells. In the salivary glands H_4R is activated by nanomolar locally produced and continuously released histamine from the ductal epithelial cells. Considering Fas-apoptosis as a key event in disease pathogenesis our findings suggest a protective role of H_4R against T-cell-mediated apoptosis in the salivary glands.

Thus, dramatically diminished expression of OCT3 transporter together with down-regulated expression of H_4R contributes to the development of the disease.

Results of Western blot and qRT-PCR experiments provided us with the data revealing underlying mechanisms of H_4R-driven protection against apoptosis. H_4R stimulation inhibited phosphorylation of JNK, a MAPK, which is considered to be a pro-apoptotic process. At the same time phosphorylation of anti-apoptotic ERK remained unchanged. These changes in the MAPK pathway should protect cells form apoptosis (Boucher *et al.* 2000). Activation of H_4R resulted in up-regulation of anti-apoptotic Bcl-XL on protein and mRNA levels, while expression of pro-apoptotic BAX protein was not changed upon H_4R stimulation in the presence of apoptosis inducers. This indicates that H_4R signaling shifts the balance of Bcl-2 family members in favor of cell survival.

Our results on anti-apoptotic low nanomolar histamine function are in agreement with those of the research group in Buenos Aires, Argentina. To our knowledge it is the only group, who is actively studying protective functions of H_4R. The mainstream of their research comprises histamine-mediated protection against radiation-induced cell damage and apoptosis *in vivo* (Martinel Lamas *et al.* 2013) and anti-proliferative activity of H_4R in cancer *in vitro* (Medina *et al.* 2011; Martinel Lamas *et al.* 2015).

Besides anti-apoptotic activity of H_4R *in vitro*, we have also demonstrated indirect evidence that H_4R regulates production of harmful IL-8 and VEGF, which are suggested to play a role in SS. High micromolar concentrations of another specific H_4R agonist ST-1006 were applied to epithelial HSG cells. This resulted in increased production of both IL-8 and VEGF by the cells. This might be explained by desensitization/down-regulation of H_4R in response to an overstimulation with high concentration of receptor's ligand, as released form mast cell-derived histamine *in vivo* or selective agonist *in vitro*. In salivary glands this response may also be aggravated by a possibly reduced withdraw of the excessive ductal epithelium histamine due to lack of major histamine transporters such as OCT3. On the other hand, pro-inflammatory cytokines like IL-8 and VEGF and others may also down-regulate H_4R expression. Both possibilities of high dose histamine-dependent and inflammatory milieu-mediated down-regulation of H_4R have been demonstrated by other members of our group (Salem *et al.* 2015). In turn, diminished activity of H_4R predisposes salivary epithelium to the development and progression of SS by creating a locally enhanced apoptotic precondition.

7.5. Regulation of H_4R expression

One of Sjögren's syndrome's marked features is the female dominance with ratio 9:1, which suggests syndrome is a sex steroid-associated disease. Recently it was reported that SS patients are androgen depleted both systemically due to adrenopause and locally in salivary glands due to a defective intracrine sex steroid synthesizing enzymatic machinery (Porola *et al.* 2008; Spaan *et al.* 2009). Furthermore, H4R expression in salivary epithelium may be reduced in patients with SS as suggested by the results of the present study. Taking together these two aspects of SS we have hypothesized that the expression of glandular H_4R is an androgen-dependent phenomenon. Our earlier unpublished results obtained using qRT-PCR with TaqMan technology showed that mRNA levels of moH_4R tend to be lower in salivary glands from orchidectomized HDC^+/HDC^+ genotype mice as compared to intact control animals (Figure 15). This preliminary observation somewhat supports the hypothesis that diminished expression of H_4R in SS may be in part due to low systemic and local levels of androgens.

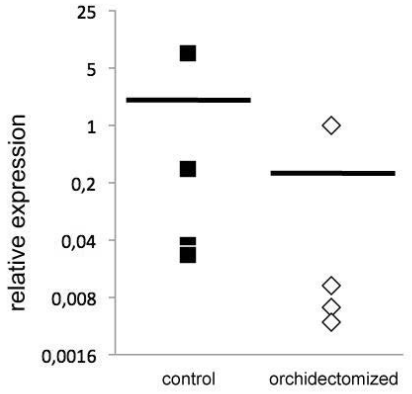


Figure 15. Relative expression of H_4R mRNA in murine salivary glands, normalized to β-actin. Number of samples in each group = 4.

Thus, based on results of this project and others it can be concluded that there are only few known mechanisms, which could lead to reduced expression of H_4R in salivary glands. At present, however, mechanisms involving a harmful local inflammatory milieu, including high levels of mast cell-derived histamine and a local/systemic androgen depletion are partly supported by results of this project.

8. SUMMARY AND CONCLUSIONS

In 1910 Sir Henry Hallett Dale discovered histamine as a contaminant of ergot generated by bacterial action. The name “histamine” originates from the Greek word “histos” (ἱστός) meaning tissue. Significance of this important biologically produced amine in medicine and biology was discovered several years later and the first clinically used antihistamine phendenzamine was synthesized and therapeutically applied in 1942 by Bernard N. Halpern.

The autoimmune alteration of major exocrine glands was discovered in 1933 by Swedish ophthalmologist Henrik Sjögren, who described sicca syndrome in his doctoral thesis at Karolinska Institutet entitled “On knowledge of keratoconjunctivitis”. Later on, the syndrome was named after the discoverer. Sjögren's syndrome still remains a disease with elusive etiology and unclear complicated pathogenesis, since many theories have been proposed to explain the causes of the disease.

Today clinically used H_1R- and H_2R-antagonists are ineffective in treatment of autoimmune diseases and have vanished from this therapy field. It has also been reported, that many cells throughout the human body produce histamine in small amounts. This finding received little attention until 1994 when the new high affinity H_4R was pharmacologically discovered in immunocompetent cells and a new era of histamine-mediated immunomodulatory effects had begun. It was hypothesized that H_4R is also implicated in Sjögren’s syndrome pathogenesis. This thesis study shows the important role of H_4R in the affected salivary glands and why diminished expression of the receptor along with locally altered histamine transport is harmful and favors development and progression of Sjögren’s syndrome. Our main conclusions of the study are:

1. Acinar and ductal epithelial cells of healthy salivary glands express H_4R on mRNA and protein levels. Based on immunohistochemical staining, H_4R expression seems to be lower in salivary glands of SS patients.

2. Salivary ductal epithelium is fully equipped with a histamine synthesizing, transporting and degradation machinery. This defines salivary epithelial cells as HDC-containing non-professional histamine producing cells, allowing H_4R

activation in auto- and paracrine manners. This finding also suggests a mast cell-derived histamine removal function of the epithelial cells, which consists of histamine up-take, subsequent intracellular degradation by HNMT and outflow into saliva. Our studies propose OCT3, a passive membrane cation exchanger, as a major histamine transporter in glandular epithelium. Expression of OCT3-based histamine transport system is drastically diminished in SS on both mRNA and protein levels. This may result in accumulation of mast cell-derived harmful high histamine amounts in extracellular space of the salivary glands in SS and exert H_1R-mediated pro-inflammatory effects. Extracellular histamine degradation in salivary glands is rather impossible due to very low, if any, expression of DAO. Thus, this study has also partly revealed the role of mast cells in SS that has since long been suspected.

3. Based on these thesis results it can be concluded that H_4R plays role in homeostatic maintenance of the glandular epithelium. It was demonstrated, that overstimulation of H_4R results in enhanced production of pro-inflammatory IL-8 and VEGF *in vitro*. We also show that H_4R selective activation results in inhibition of Fas-apoptosis, which is considered as a key event in SS pathogenesis. Underlying mechanisms of H_4R-mediated anti-apoptotic activity comprise inhibition of JNK MAPK pathway and shift in the balance within Bcl-2 family proteins in favor of cell survival by up-regulating Bcl-XL anti-apoptotic protein.

4. This thesis project also aimed at preliminarily searching the mechanisms which might down-regulate H_4R expression in salivary glands. It seems, that locally increased pro-inflammatory cytokine production inside the lymphocytic foci and accumulation of mast-cell derived histamine and local/systemic androgen deficiency may be proposed as suppressive candidates for H_4R expression. These preliminary findings were obtained from mouse salivary glands and supported by scant *in vitro* studies by other investigators. Further investigation is required on this matter.

5. All presented data are novel reported findings and do not reproduce earlier performed and published experiments of others. New roles for nanomolar histamine have been proposed. The failure of those H_4R-driven mechanisms can

contribute to SS pathogenesis. For the future we believe that newly synthesized low molecular weight H_4R agonists along or together with mast cell stabilizers might provide alternatives to expensive widely used biologicals for the treatment of autoimmune diseases, including Sjögren's syndrome.

9. SUMMARY AND CONCLUSIONS in RUSSIAN

Гистамин был открыт в 1910 году сэром Генри Галлеттом Дейлом как побочный продукт бактериальной ферментации спорыньи. Термин «гистамин» происходит от греческого слова *гистос* (ἱστός), что значит *ткань*. Годами позже была открыта его биологическая и медицинская значимость, а первый антигистаминовый препарат фендензамин был синтезирован и применен в лечебной практике Бернардом Н. Халперном в 1942 году.

Аутоиммунное поражение основных экзокринных желез было открыто в 1933 году шведским офтальмологом Хенриком Шегреном. Он описал синдром сухости в своей докторской диссертации «К вопросу о кератоконъюнктивите», которую защитил в Каролинском университете. Позднее синдром был назван в честь своего первооткрывателя. Много теорий было предложено для объяснения причин синдрома Шегрена (СШ), но и по сей день он остается заболеванием с ускользающей от исследователей этиологией и с неясным патогенезом.

Используемые сегодня H_1- и H_2-антигистаминовые препараты неэффективны в лечении аутоиммунных заболеваний, и поэтому не используются в данном терапевтическом назначении. Исследования показывают, что гистамин синтезируется в малых количествах по всему организму человека. Это открытие не получало заслуженного внимания до тех пор, пока в 1994 году в иммунокомпетентных клетках не был открыт новый высокоаффинный рецептор H_4R, что положило начало новой иммуномодуляторной гистаминовой эре. Было предположено, что H_4R вовлечен в патогенез синдрома Шегрена. Данная диссертационная работа показывает важную роль этого рецептора в пораженных слюнных железах и объясняет, чем сниженная экспрессия рецептора, наряду с локально поврежденным транспортом гистамина, является вредоносным и способствует развитию и прогрессу заболевания. Основные выводы работы:

1. Ацинарные и дуктальные клетки здорового эпителия слюнных желез экспрессируют H_4R как на мРНК, так и на белковом уровнях. Основываясь на результатах иммуногистохимии, экспрессия H_4R представляется сниженной в слюнных железах больных СШ.

2. Канальцевый эпителий слюнных желез полностью оснащен механизмами, необходимыми для синтеза, транспорта и деградации гистамина ферментами и транспортерами, в частности гистидиндекарбоксилазой (HDC), гистамин N-метилтрансферазой (HNMT) и транспортером органических катионов-3 (OCT3). Это определяет HDC-содержащие клетки эпителия как непрофессиональные гистамин-продуцирующие клетки и дает возможность для аутокринной и паракринной активации H_4R. На основании данного положения также можно предположить, что слюнные эпителиальные клетки выполняют функцию утилизации сравнительно больших объемов гистамина, высвобожденного в результате дегрануляции тучных клеток, путем его захвата и деградации ферментом HNMT с дальнейшим выведением со слюной. Наши исследования предлагают ведущую роль OCT3 в транспорте гистамина в эпителии слюнных желез. Экспрессия этого пассивного мембранного обменника катионов в слюнных железах больных СШ значительно снижена на уровнях белка и мРНК. В свою очередь, это может служить причиной накопления высвобожденного из тучных клеток гистамина высоких концентраций в ткани слюнных желез больных СШ с последующим усилением местных H_1R-опосредованных воспалительных реакций. Согласно нашим данным, внеклеточная деградация гистамина в слюнных железах невозможна в результате отсутствия фермента диаминооксидазы (DAO). Таким образом, данное исследование также частично описывает вовлеченность тучных клеток в СШ, чья роль в патогенезе данного заболевания подозревалась уже довольно давно.

3. Основываясь на результатах данной работы, можно заключить, что H_4R играет роль в поддержании гомеостаза эпителия слюнных желез. Было продемонстрированно, что чрезмерная стимуляция H_4R приводит к увеличению продукции провоспалительных цитокинов IL-8 and VEGF *in vitro*. Также было показано, что селективная активация H_4R ингибирует Fas-опосредованный апоптоз, ключевое событие в патогенезе СШ. Молекулярные механизмы H_4R-опосредованного антиапоптотического эффекта включают в себя ингибирование JNK MAPK сигнального пути и

сдвиг баланса внутри белковой группы Bcl-2 с преимущественным увеличением синтеза антиапоптотического белка Bcl-XL.

4. Данная диссертационная работа также ставила задачи по поиску причин снижения экспрессии H_4R в слюнных железах у больных СШ. С определенной долей вероятности можно предположить, что локально увеличенная продукция провоспалительных цитокинов в фокусных скоплениях лимфоцитов и аккумуляция высвобожденного из тучных клеток гистамина а также локальный и системный дефицит андрогенов, являются основными супрессивными кандидатами для подавления синтеза и экспрессии H_4R. Легшие в основу данного вывода наблюдения были получены в экспериментах с мышиными слюнными железами и подкрепляются небольшим количеством сторонних исследований *in vitro*. Более подробное объяснение данных наблюдений требуют дальнейших исследований.

5. Все представленные данные являются новыми и не повторяют ранее проведенных и опубликованных результатов экспериментов. Предложены новые роли гистамина наномолярных концентраций. Нарушения описанных H_4R-опосредованных механизмов могут быть вовлечены в патогенез СШ. Мы полагаем, что новые синтетические низкомолекулярные агонисты H_4R совместно со стабилизаторами тучных клеток в будущем могут служить альтернативой повсеместно используемым дорогостоящим биологическим препаратам в лечении аутоиммунных заболеваний, включая синдром Шегрена.

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